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HISTOCHEMICAL CHANGES IN THE SUPRARENAL GLAND OF THE ADULT MALE RAT

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The object of the present work has been to observe the changes occurring in the suprarenal gland of the adult male rat after the administration of pituitary adrenotropic hormone (PATH) and extract of suprarenal cortex (Eschatin).†

MATERIAL AND METHODS

Seventy-eight adult male rats of the Wistar strain were used, of which forty-six were treated, twenty were untreated and twelve were controls. In experiments lasting longer than 6 hr., PATH and cortical extract were injected subcutaneously. In experiments lasting 6 hr. or less, it was thought desirable to have a precise starting-point, and injections were made into the femoral vein under brief ether anaesthesia, thus obviating a possible source of confusion due to varying rates of absorption after subcutaneous administration. It had been hoped that with brief anaesthesia (10 min.) the adaptational reaction noted by Selye (1937) would be of only moderate intensity, and that additional changes would follow the administration of PATH. However, the interpretation of these further changes proved difficult. Six control rats anaesthetized for 10 min. each, and then killed in pairs 1, 3 and 6 hr. later, all showed a characteristic adaptation ('alarm') syndrome. Where the survival period was longer than 6 hr., and the injection was made without anaesthesia, the changes in the suprarenal cortex could be interpreted as an uncomplicated response to PATH or cortical extract. In six control rats which were given subcutaneous injections of normal saline, and killed in pairs 3, 6 and 12 hr. later, no significant changes were noted in the cortex.

Dose and duration of experiment. PATH was given to each of twenty-eight rats in a single dose of two sudanophobic units (Reiss, Balint, Oestreicher & Aronson, 1936) per 100 g. body weight, and the animals were killed from 1 hr. to 3 days after injection. Eschatin was injected daily into fourteen rats, in a single dose of 5 dog units (Harrop, Pfiffner, Weinstein & Swingle, 1932) per 100 g. body weight, for times ranging from 1 to 14 days and killed 24 hr. after the last injection. Four further rats were given a daily injection of eschatin for 3 days, and killed 12 hr. after a single injection of PATH on the 4th day.

HISTOCHEMICAL TECHNIQUE

The animals having been killed by coal gas, the suprarenals were removed immediately and fixed in formol-calcium (10% formol in distilled water, with the addition of 1% calcium chloride) for 3 days, and washed in running tap water for 24 hr.; they were then placed in a 25% solution of gelatin (with a trace of phenol) in a desiccator, and

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† For supplies of adrenotropic hormone (Cortrophin, Organon) we are indebted to Organon Laboratories Ltd. Extract of suprarenal cortex (Eschatin) has been provided by the courtesy of Dr Stanley White, Parke, Davis and Co.

left in an incubator at 37° C. for 30 hr. After this embedding in gelatin, the block was placed in 10 % formol-calcium with cadmium chloride (1 %) added, and kept in the refrigerator at about 2° C. for 3 days before cutting. This technique produces a block with about the consistency of celloidin whatever the external temperature. Sections were cut at 10 μ and mounted on gelatin-covered slides. The following stains were employed, in the manner described.

Sudan black. Sections were rinsed for a few seconds in 50 % and 70 % alcohol, then stained for 3 min. in a saturated solution of Sudan black (Gurr) in 70 % alcohol. They were then washed in two changes of 50 % alcohol and mounted in glycerine jelly. The technique for staining, as also for fixation and embedding, is substantially that employed by Baker (1945).

Phenylhydrazine. At first 2:4-dinitrophenylhydrazine sulphate was employed (see Yoffey & Baxter, 1948), but it was found not to be so precise as 2:4-dinitrophenylhydrazine hydrochloride, and was therefore abandoned in favour of the latter. Sections were treated for 3–4 hr. in a saturated solution of 2:4-dinitrophenylhydrazine hydrochloride in equal volumes of 2 N/HCl and absolute alcohol. They were then washed in two changes of a fluid consisting of equal parts of absolute alcohol and concentrated hydrochloric acid, allowing 5 min. in each, and mounted in glycerine jelly. The employment of hydrochloric acid was suggested to us by Prof. Wilson Baker, in order to dissolve out 2:4-dinitrophenylhydrazine hydrochloride and leave only the 2:4-dinitrophenylhydrazones.

Plasmal reaction. Sections were well washed in running tap water and then in distilled water. They were treated for 3 min. with a saturated solution of mercuric chloride in water, after which they were immersed in Schiff's reagent (fuchsin-sulphurous acid) for 15 min. After three washings in sulphurous acid water, each lasting 30 sec., they were washed in distilled water and mounted in glycerine jelly.

Schultz reaction. Sections were treated for 2 days with 2·5 % aqueous iron alum at 37° C. They were then allowed almost to dry on the slide, and covered with a drop of equal parts of concentrated sulphuric acid and acetic anhydride, the cover-slip being sealed with paraffin wax. The sealing is important, for it enables the specimen to be kept for 24–48 hr. whereas if not sealed it becomes useless after a few hours.

When weather permitted, sections after treatment with iron alum were exposed to bright sunshine for 3 days, as recommended by Schultz (1924). The combination of iron alum and sunlight was found to give more effective coloration than either alone.

THE SIGNIFICANCE OF THE HISTOCHEMICAL REACTIONS EMPLOYED

The phenylhydrazine reaction. The phenylhydrazine reaction was first applied to the suprarenals by Bennett (1940), who believed that it was a specific test for corticosterones. Using phenylhydrazine hydrochloride, he worked with thick sections, and observed the reaction with incident light. The specificity of the reaction was questioned by Gomori (1942), Albert & Leblond (1946) and others. They maintained that the substances which stained with phenylhydrazine were the same as those which gave the plasmal reaction, and that the phenylhydrazine reaction gave no additional information.

The plasmal reaction. The plasmal reaction was first used by Feulgen & Voit (1924) and has been extensively employed in recent years. According to Thannhauser & Schmidt (1946), the reaction indicates acetals of fatty aldehydes (chiefly stearic and palmitic) bound with colamine glycerophosphate. 'Plasmalogen', the substance which becomes transformed into 'plasmal' to give the reaction, is associated in the tissues with phospholipids, but not infrequently the extent of the area staining with Sudan black differs markedly from that staining with Schiff's reagent.

In general it is true that tissues—including fat—which give a positive plasmal reaction also give a positive phenylhydrazine reaction, though in the case of the suprarenal cortex the plasmal and phenylhydrazine areas may differ markedly in extent. Whatever the precise nature of the substance or substances stained by the plasmal and phenylhydrazine reactions, their distribution seems to vary in accordance with functional changes in the suprarenal gland in a manner which may throw some light on the gland's function.

The Schultz reaction. The Schultz reaction was introduced in 1924, as an application to sections of the Liebermann-Burchardt colour test for sterols (see Fieser, 1936). According to Schultz the cholesterol has first to be oxidized by sunlight or iron alum to 'oxycholesterol', concerning the chemical nature of which Windaus, when consulted by Schultz (1924), was somewhat guarded, but which presumably corresponds to the dihydroxycholesterol of our terminology. According to Fieser (1936) 'the colour reactions distinguish sharply the unsaturated from the saturated sterols...', and the question which obviously arises is whether the Schultz reaction is specific for cholesterol, or whether it is given by any other unsaturated sterols. On comparing the Schultz coloration with the colour reactions of some pure steroid hormones, it was noted that, whereas the Schultz reaction gives a green colour, progesterone crystals on a slide gave a bright yellow when treated with the Schultz reagents, as did oestrone; desoxycorticosterone acetate (Doca) gave a dull brick with a slight bluish tinge, and even androsterone, which is saturated except for the carbonyl group, became a brownish yellow.

Recently Everett (1947) has concluded that the typical Schultz colour response is limited to the diols formed from cholesterol by mild oxidation procedures. Everett further noted that when cholesterol was mixed in gelatin with lecithin and other fats, the Schultz reaction ceased to be positive at a cholesterol concentration lower than 1:10. The reaction, with a little care, is one of considerable precision. Even within a single cell it can be shown that its distribution is not uniform, for it is limited to the cytoplasm and does not involve the nucleus (Pl. 1, fig. 5). This being the case, it appears certain that the occurrence in the gland as a whole of Schultz-positive and Schultz-negative zones accurately reflects the distribution of the cholesterol derivatives which give the reaction.

The observation of changes in the distribution of cholesterol within the suprarenal gland is of considerable interest. It has been known for some time (see review by Tepperman, Engel & Long, 1943) that, after feeding diets rich in cholesterol, the gland may show a marked increase in its cholesterol content and even undergo hypertrophy. Recent work (Bloch, 1945; Sayers, Sayers, Liang & Long, 1946) indicates that cholesterol accumulates in the suprarenals as the raw material out of which steroid hormones are made. It would not be unreasonable, then, to expect

that marked variation in the secretory activity of the suprarenal cortex should be accompanied by appreciable changes in its cholesterol content on a scale sufficient to allow of histochemical demonstration.

In general distribution and intensity, the Schultz, phenylhydrazine and plasmal reactions, and the Sudan black stain, show a rough parallelism. As far as concerns the Schultz reaction and sudanophile lipoids, this is in accord with the view that birefringent crystals in the suprarenal cortex are composed of cholesterol, for Yoffey & Baxter (1948) noted that these crystals, though not themselves sudanophile are most abundant in the region of maximal sudanophilia. The correspondence of the plasmal and sudanophile zones fits in with the view of Thannhauser & Schmidt (1946) concerning the fatty nature of plasmalogen. Phenylhydrazine no doubt does form phenylhydrazones with cortical steroids, and possibly with the fatty aldehydes which yield the plasmal reaction. In the present work it has been found to stain lipoids generally in spite of attempts to dissolve out excess phenylhydrazine by hydrochloric acid.

It must, however, be noted that the plasmal and Sudan black zones do not always correspond, nor do the plasmal and phenylhydrazine zones. In the present state of our knowledge it is only possible to draw attention to these facts without being able to explain them adequately. With regard to the plasmal and Sudan black zones, several experiments showed zones which stained with Sudan black but did not give a plasmal reaction. Presumably the fatty aldehydes can disappear as a result of cortical stimulation while sudanophile lipoids are still present.

Pl. 1, figs. 9 and 10 illustrate a gland in which the phenylhydrazine and plasmal zones were markedly different, the former being much more extensive than the latter, and occupying most of the depth of the zona reticularis. Whatever the detailed chemical explanation, such experiments indicate that the criticisms of the phenylhydrazine reaction advanced by Gomori (1942) and others, at any rate in so far as it was equated with the plasmal reaction, are not altogether valid. In a personal communication sent to us immediately before publication, Dr J. Danielli reports a recent conclusion by Boscott, Mandl, Danielli & Shoppee (1948, unpublished), that 'the aldehyde reactions obtained by using the plasmal technique on the suprarenal cortex show the presence of some compound other than desoxycorticosterone or allied ketones'. With this the present experiments are in full agreement, for we find that, in different functional states of the cortex, either the plasmal-positive zone may sometimes be markedly greater than that which gives a phenylhydrazine reaction, or vice versa.

RESULTS

A. The normal suprarenal

In the suprarenal of the normal male rat the plasmal, phenylhydrazine, Sudan black, and Schultz colorations extend through the zona fasciculata and reticularis (Pl. 1, figs. 1–5) as far as the medulla, from which they are absent, except for nerve fibres and islets of cortical tissue which may sometimes be found in the medulla. The stain is usually strongest in the outer part of the zona fasciculata, and less dense in the reticularis, giving rise to a characteristic gradient. As a rule the transition between fasciculata and reticularis is not abrupt, except perhaps occasionally in the case of the plasmal and phenylhydrazine reactions.

The zona glomerulosa is not always well marked, though when it is present it may be the most heavily stained part of the cortex, with a clear subglomerular zone between it and the outer part of the zona fasciculata. The marked variability of the zona glomerulosa as compared with the remainder of the cortex would accord with the views of Greep & Deane (1947), in so far as they suggest different functions for glomerulosa and fasciculata. In view of this variability it becomes difficult to define what is a normal glomerulosa. Frequently the distribution of staining material is patchy; occasionally a varying length of glomerulosa may be clear and free from stain, while the remainder may be fully stained. In view of the possibility that the conditions of cage life (three to six rats in a cage) might repeatedly stimulate cortical secretion, eliciting at intervals Selye's (1937, 1946) alarm reaction, it was decided to try the effect of isolation. Three rats were kept in separate cages for 24 hr., and then killed; the suprarenals of these rats showed in two cases full staining of the glomerulosa, in the third case almost complete absence of staining. Three further rats were then isolated for 40 days, and precisely the same result was obtained, namely two with full glomerulosal staining, one with complete absence of staining. It seems reasonable then to infer that glomerulosal discharge (of material stainable with the plasmal, phenylhydrazine, Schultz and Sudan black reactions) whatever its cause is not a stress reaction. The zona fasciculata and zona reticularis, on the other hand, under the usual conditions of cage life, never show a discharge, though they can be made to do so in response to a variety of stimuli. Because of the variability of the glomerulosal staining reactions, even in the normal animal, attention in the present paper has been directed mainly to the zona fasciculata and reticularis, and only incidental observations have been made on the glomerulosa.

B. The effect of pituitary adrenotropic hormone (PATH)

(1) *Schiff's reagent, Phenylhydrazine, and Sudan black*

As already noted the results from 1–6 hr. after injection are complicated by the fact that these animals were subjected to brief ether anaesthesia. The effects of anaesthesia are being investigated in further detail, and will not be described here. With this qualification, the changes after PATH injection (plasmal, phenylhydrazine, Sudan black) may be summarized as follows:

One hour after injection. Two experiments. In both there was depletion of the reticularis with all three reagents, while the zona fasciculata appeared normal.

Three hours after injection. Two experiments. In both the staining of the outer fasciculata was intensified, but whereas in one the reticularis was depleted, in the other it was not.

Six hours after injection. Five experiments. In two experiments the only obvious change was in the reticularis, which in one case showed slight weakening, in another marked depletion. In two experiments there was intensified staining of the fasciculata with weakening of the reticularis. The fifth experiment showed what we have termed a reversal effect, namely weakening of the fasciculata and intensified staining of the reticularis.

Twelve hours after injection. Six experiments. Three appeared normal. One showed marked depletion of the reticularis, and between it and the fasciculata an

intermediate zone most evident with Sudan black. The remaining two showed weakening of the reticularis, with moderate intensification of the fasciculata in one of them.

Twenty-four hours after injection. Six experiments. Two were practically normal. In one, depletion of the reticularis with phenylhydrazine and Sudan black, but not with Schiff's reagent. In one, a marked reversal effect with Schiff's reagent, but not with phenylhydrazine. One showed depletion of the reticularis with Schiff's reagent, but not with phenylhydrazine. One showed a slight reversal effect with Schiff's reagent.

Forty-eight hours after injection. Five experiments. Two showed marked depletion of the reticularis with both the plasmal and phenylhydrazine reactions, and one of these (Pl. 1, figs. 9–11) showed a well-marked intermediate zone between fasciculata and reticularis, much wider with phenylhydrazine than with Schiff's reagent. Two were almost normal, though in one there was a narrow juxtamedullary zone completely depleted, and in the other a similar zone with weak staining. The appearances suggest that the stainable matter extends into the reticularis from the fasciculata. One showed intensification of the fasciculata as well as weakening of the reticularis.

Three days after injection. Two experiments. One appears normal. One has an intensely stained narrow band in the fasciculata, and a large weakly staining reticularis.

(2) *The Schultz reaction after administration of PATH*

Six hours after the administration of PATH the Schultz reaction demonstrates marked changes (Pl. 1, fig. 6). It disappears altogether in the reticularis, and occupies only a narrow and weakly staining zone in the fasciculata. At 48 hr. after injection (Pl. 1, fig. 12) cholesterol is present in amounts which are nearer the normal, though by the other tests employed the cortex is still far from normal.

Discussion of PATH results

Plasmal and phenylhydrazine zones do not always coincide. There is frequently a difference in extent between the plasmal and phenylhydrazine zones. In two 48 hr. experiments, for example, the reticularis showed marked depletion with Schiff's reagent, much less with phenylhydrazine (Pl. 1, figs. 9, 10). Cases also occur where the zone of phenylhydrazine depletion is wide, that of plasmal depletion narrow. It is important to compare sections taken through the middle of the gland, and to avoid oblique cuts through the cortex. In the present experiments the procedure adopted was to cut fifteen such sections at 10 μ and discard the remainder of the gland.

Functional significance of plasmal and phenylhydrazine reactions. That the substances which give the plasmal and phenylhydrazine reactions frequently do not coincide in extent would accord with the original interpretation placed upon the phenylhydrazine reaction by Bennett (1940). On the other hand, the fact that fat can give a well-marked phenylhydrazine coloration suggests some caution in accepting this interpretation. On the basis of the present series of experiments, all that can be legitimately inferred is that, in stimulation of the cortex by PATH,

characteristic changes are produced in both the plasmal and phenylhydrazine reactions, so that one can decide with some degree of certainty whether the cortex is in the resting state or not.

Does a single dose of PATH induce cyclic changes? The fact that there can be fairly rapid depletion of the reticularis occurring within the first 6 hr., then seemingly normal cortex in some cases at 12 hr., with depletion again in evidence in some instances at 24 hr. and at 48 hr., suggests the possibility that a single dose of PATH may produce cyclic changes, with repeated emptying and filling of the reticularis. This is obviously linked with a number of other questions the answers to which are not known, such as: How long can PATH remain unaltered in the blood? Can some of it be used to stimulate cortical secretion, while the remainder circulates unchanged in the blood to act on the cortex when the first wave of secretion is over? Is there a quantitative relationship between PATH and the suprarenal cortex? Recently introduced methods for the estimation of cortin in the blood (Hemphill & Reiss, 1947) may provide an important link in the chain of evidence. It is interesting to note that even 3 days after a single injection of PATH the cortex may not have returned to its resting state.

The functional zoning of the cortex. Arnold (1866) on morphological grounds first introduced the traditional zoning of the cortex into glomerulosa, fasciculata and reticularis. In the rat a narrow but definite subglomerular zone also exists. Bennett (1940) suggested in the cat a grouping into four zones—presecretory, secretory, postsecretory and senescent. In the rat, apart from the glomerulosa which would correspond to the presecretory zone of Bennett, the present experiments do indicate three further zones in the cortex, making a total of four zones in all. However, the zone which Bennett terms senescent appears to be one which may repeatedly fill with stainable matter and become depleted, so it may conceivably play a rather more active part in cortical functioning than the term 'senescent' would imply. If the stainable matter is associated with cortical hormones, then the zona reticularis could well be a storage zone for cortical secretion, which it might rapidly discharge when needed. If, on the other hand, the reticularis is a secretory zone, its cholesterol content would indicate much less secretory activity than in the fasciculata. In one of the rats isolated for 40 days there was marked increase of stainable matter in the reticularis, and this would agree with the idea that it was a storage zone in which secretion had accumulated under stress-free conditions. It should perhaps be noted that a division of the cortex into fixed zones may not quite correspond with the facts, since under varying functional conditions the cortex may show a good deal of plasticity. It may even be that the different zones are to a considerable extent interchangeable.

The outer part of the fasciculata, from its rich cholesterol content, would appear to be secretory. The reticularis may be either weakly secretory or a storage zone. The purpose of the intermediate zone is not quite clear, but from observing differences in its width, it seems conceivable that it may represent the gradual extension of stainable matter, possibly secretion, from the outer to the inner zone (Pl. 1, figs. 9–11).

Variability of results. In spite of apparent uniformity in dosage and other experimental factors, the response of the suprarenal gland in the various animals was not

constant. We are unable to account for this, and can only speculate that either the glands were not all in the same functional condition at the outset, or else that in some animals unappreciated differences in the conditions prevailing at the time of injection may have evoked additional responses, of Selye's (1946) adaptational type.

Significance of cholesterol changes. The diminution in the cholesterol content of the fasciculata may be attributed to its conversion into steroid hormones which are discharged into the blood stream, and presumably fresh cholesterol would be taken up from the blood by the cells of the cortex to replace that utilized in the elaboration of hormones. If hormone secretion is stimulated, its rate of formation and discharge could conceivably exceed that of cholesterol replacement so that a very active cortex might be almost devoid of cholesterol. This view is supported by the finding, already noted, that 6 hr. after a single injection of PATH the cholesterol content of the cortex shows a marked diminution. Conversely, if the discharge of hormone is retarded, the rate of cholesterol deposition in the cortex might either be unchanged, in which case it would gradually accumulate in the gland, or else the cholesterol would only be taken up as required for conversion into the hormone, in which case it would not show a great increase. In order to determine whether the cholesterol taken up by the suprarenal cortex was (*a*) independent of, or (*b*) ran parallel with the secretion of steroid hormones, a number of rats were given repeated injections of cortical extract. The effect of this would presumably be to make it unnecessary for the cortex to secrete its own hormones, and if (*a*) were true cholesterol would accumulate in the cortex, whereas if (*b*) were the case it would not.

C. The effect of cortical extract

The effect of repeated administration of cortical extract was to cause intensified staining by all the methods employed, in both the glomerulosa and the remainder of the cortex, during the first 3 days. Subsequently there was a gradual weakening, which by 14 days was very marked. The simplest explanation of this sequence of events is that for the first 3 days the gland was functioning normally, and that steroid hormones and their precursors accumulated in the cortex. When the administration of cortical extract was continued beyond this stage, the cortical cells underwent degenerative changes. The Schultz response at the end of 3 days is very heavy both in the glomerulosa and the fasciculata. It never becomes very intense in the reticularis, though as it accumulates in the fasciculata the latter zone may encroach considerably on the former.

D. The effect of cortical extract followed by PATH

If cortical extract is given daily for 3 days, the accumulation of cholesterol is intense (Pl. 1, fig. 7). If then on the fourth day PATH is given, this accumulated cholesterol quickly disappears from the fasciculata and the reticularis, but not from the glomerulosa (Pl. 1, fig. 8). As far as the fasciculata is concerned, this finding fits in well with the view that cholesterol is one of the raw materials for the manufacture of cortical hormones. The glomerulosa, on the other hand, does not respond in the same way to PATH. It has already been noted, on the basis of observations on isolated animals, that depletion of the glomerulosa does not appear to be a stress

reaction, and the further observation that PATH does not influence its cholesterol content even when the latter is heavy seems to confirm this.

SUMMARY AND CONCLUSIONS

1. The reactions of the suprarenal glands of adult male Wistar rats to pituitary adrenotropic hormone (PATH) and extract of suprarenal cortex have been studied, using Sudan black staining, as well as the plasmal, phenylhydrazine and Schultz reactions.

2. Changes in the glomerulosa appear to be independent of those in the remainder of the cortex.

3. The view is advanced that, while the fasciculata is secretory in function, the reticularis may be a storage zone.

4. An intermediate zone between the fasciculata and reticularis is sometimes very evident.

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REFERENCES

ALBERT, S. & LEBLOND, C. P. (1946). The distribution of the Feulgen and 2, 4-dinitrophenylhydrazine reactions in normal, castrated, adrenalectomized and hormonally treated rats. *Endocrinology*, **39**, 386–400.

ARNOLD, J. (1866). Beitrag zu der feineren Structur und dem Chemismus der Nebennieren. *Virchows Arch.* **35**, 64–107.

BAKER, J. R. (1945). The structure and chemical composition of the Golgi element. *Quart. J. micr. Sci.* **85**, 1–71.

BENNETT, H. S. (1940). The life history and secretion of the cells of the adrenal cortex of the cat. *Amer. J. Anat.* **67**, 151–227.

BLOCH, K. (1945). The biological conversion of cholesterol to pregnanediol. *J. biol. Chem.* **157**, 661–666.

BOSCOTT, R. J., MANDL, A., DANIELLI, J. & SHOPPEE, C. W. (1948). Unpublished.

EVERETT, J. W. (1947). Hormonal factors responsible for deposition of cholesterol in the corpus luteum of the rat. *Endocrinology*, **41**, 364–377.

FEULGEN, R. & VOIT, K. (1924). Über einen weitverbreiteten festen Aldehyd. Seine Entstehung aus einer Vorstufe, sein mikrochemischer und mikroskopisch-chemischer Nachweis und die Wege zu seiner präparativen Darstellung. *Pflüg. Arch. ges. Physiol.* **206**, 389–410.

FIESER, L. F. (1936). *The Chemistry of Natural Products Related to Phenanthrene.* New York. Reinhold Publishing Corporation.

GOMORI, G. (1942). Histochemical reactions for lipoid aldehydes and ketones. *Proc. Soc. exp. Biol., N.Y.*, **51**, 133–134.

GREEP, R. O. & DEANE, H. W. (1947). Cytochemical evidence for the cessation of hormone production in the zona glomerulosa of the rat's adrenal cortex after prolonged treatment with desoxycorticosterone acetate. *Endocrinology*, **40**, 417–425.

HARROP, G. A. Jr., PFIFFNER, J. J., WEINSTEIN, A. & SWINGLE, W. W. (1932). A biological method of assay of the adrenal cortical hormone. *Proc. Soc. exp. Biol., N.Y.*, **29**, 449–451.

HEMPHILL, R. E. & REISS, M. (1947). Regulation of endogenous cortin production. *Endocrinology*, **41**, 17–20.

REISS, M., BALINT, J., OESTREICHER, F. & ARONSON, V. (1936). Zur morphogenetischen Wirkung und biologischen Eichung des kortikotropen Wirkstoffes. *Endokrinologie*, **18**, 1–10.

SAYERS, G., SAYERS, M. A., TSAN-YING LIANG & LONG, C. N. H. (1946). The effect of pituitary adrenotropic hormone on the cholesterol and ascorbic acid content of the adrenal of the rat and the guinea pig. *Endocrinology*, **38**, 1–9.

Schultz, A. (1924). Eine Methode des mikrochemischen Cholesterinnachweises am Gewebsschnitt. *Zbl. allg. Path. path. Anat.* **35**, 314–317.

Selye, H. (1937). Studies on adaptation. *Endocrinology*, **21**, 169–188.

Selye, H. (1946). The general adaptation syndrome and the diseases of adaptation. *J. clin. Endocrin.* 6, 117–230.

Tepperman, J., Engel, F. L. & Long, C. N. H. (1943). A review of adrenal cortical hypertrophy. *Endocrinology*, **32**, 373–402.

Thannhauser, S. J. & Schmidt, G. (1946). Lipins and lipidoses. *Physiol. Rev.* **26**, 275–318.

Yoffey, J. M. & Baxter, J. S. (1948). The formation of birefringent crystals in the suprarenal cortex. *J. Anat., Lond.*, **81**, 335–342.

EXPLANATION OF PLATE

All the illustrations are photomicrographs of frozen sections 10μ in thickness. *A* = glomerulosa, *B* = subglomerular zone, *C* = fasciculata, *R* = reticularis, *M* = medulla, *I* = intermediate zone, *F* = Pericapsular fat.

Note. Some of the lettering is slightly out of place. In Fig. 1 the letter *B*, in Fig. 3 the letters *A* and *B*, in Fig. 6 the letter *A*, in Fig. 7 the letters *A*, *B* and *C*, in Fig. 8 the letters *B* and *C* all should be slightly nearer the surface of the gland, to indicate the layers correctly. In Fig. 9 the lettering should be reversed so as to form a mirror image of Fig. 10.

Figs. 1–4. Suprarenal of normal adult male rat, showing the plasmal (Fig. 1) and phenylhydrazine (Fig. 2) reactions, Sudan black stain (Fig. 3), and the Schultz reaction (Fig. 4). Note the glomerulosa and subglomerular zone, and the moderately marked gradient of staining in the remainder of the cortex, the staining being slightly more intense in the fasciculata, and weaker nearer the medulla. There is no widespread staining of the medulla, though in Fig. 4 the medulla has an islet of cortical tissue. × 50.

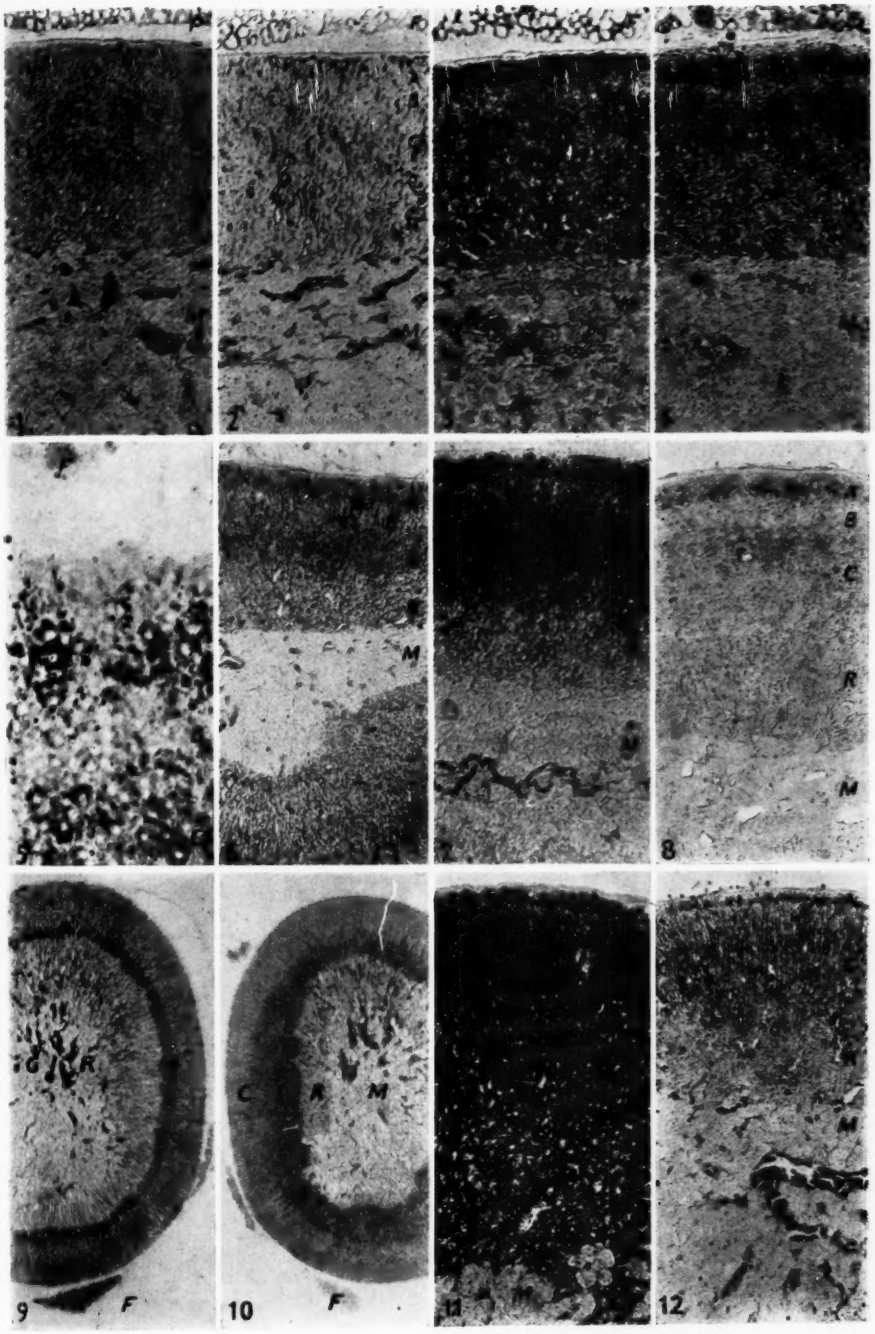
Fig. 5. High-power view of part of Fig. 3, showing the localization of the Schultz reaction to the cytoplasm of the cell, the nucleus being Schultz negative. × 200.

Fig. 6. Diminution of the Schultz positive zone 6 hr. after a single intravenous injection of PATH. × 35.

Fig. 7. Schultz reaction in the suprarenal of a rat which had been given daily injections of cortical extract for 3 successive days, and was killed on the 4th. Compare with the Schultz reaction in the normal gland (Fig. 4), and note that the reaction is now much heavier both in glomerulosa and remainder of cortex, that the strongly Schultz positive zone is wider, extending well into the reticularis from the fasciculata. × 50.

Fig. 8. Schultz reaction in the suprarenal of a rat which had been given daily injections of cortical extract for 3 successive days, then on the 4th received a single dose of PATH and was killed 12 hr. later. Compare with Fig. 7, and note the marked diminution in cholesterol content of fasciculata and reticularis. × 50.

Figs. 9–12. Suprarenal gland of rat killed 48 hr. after a single injection of PATH. Fig. 9, plasmal reaction; Fig. 10, phenylhydrazine reaction; Fig. 11, Sudan black stain; Fig. 12, Schultz reaction. The photographs are from a set of fifteen sections taken through the middle of the gland. The plasmal reaction is positive over a narrower zone than the phenylhydrazine. Fig. 9 shows a well-marked intermediate zone, and Fig. 10 a broadening of the intermediate zone so as to give a reversal of the usual colour gradient, with the reaction more intense in the reticularis than the fasciculata. Fig. 11 shows a positive Sudan black stain in a region which gives a negative plasmal and phenylhydrazine reaction. (Figs. 9 and 10, × 17·5; Fig. 11, × 50; Fig. 12, × 35.)



YOFFEY AND BAXTER—CHANGES IN SUPRARENAL GLAND OF ADULT MALE RAT

AN INJECTION APPARATUS INCORPORATING A MAXIMUM PRESSURE SAFETY DEVICE

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The use of liquid Latex or Vinylite Resin for making corrosion specimens is accompanied by the disadvantage that syringes of the metal to metal or metal to glass types have a great tendency to jam after the injection of a few millilitres of the mass. Several workers have overcome this difficulty by using apparatus of their own design, all basically similar and depending on compressed air or gas of some description for injection of the mass. Such is the apparatus of Narat, Loef & Narat (1936), Tobin (1947), and Trueta, Barclay, Daniel, Franklin & Prichard (1947).

Such technique is undeniably superior to the use of the syringe, even of the glass to rubber or fibre type, for large specimens giving, as it does, a comparatively even pressure over a considerable period. Nevertheless, some slight pressure fluctuation seems in our experience to occur when using this apparatus. We have succeeded in eliminating such fluctuation, providing at the same time a maximum pressure safety valve by inserting into the usual circuit, in lieu of the Winchester bottle or other reservoir, a simple device as follows (Text-fig. 1).

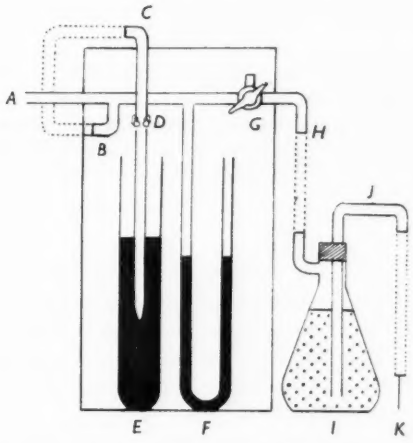

Fig. 1. Rubber tubing connexions in broken outline.

Compressed air enters at *A* and fills the apparatus. A T-piece *B* gives access via a length of rubber tubing to a length of glass tubing *C*. This tube *C* is held by a clip *D* through which it can slide up and down, and it dips into a large tube *E* containing mercury. According to the depth of immersion of tube *C* into the mercury so the compressed air has greater or less difficulty in escaping through the mercury, and

the pressure in the apparatus is registered by manometer *F*. At *G* is a three-way stopcock which allows the compressed air to flow via tube *H* to injection mass container *I*, or shuts off tube *H* from the rest of the apparatus, or allows the pressure of gas in tube *H* and container *I* to escape into the atmosphere, thus ensuring an immediate cessation of the flow of the injection mass from *I* through *J* to needle or cannula *K* whenever desired.

This apparatus has been in use for almost two years and has proved eminently satisfactory for the injection of corrosion specimens, the injection of indian ink and various radio-opaque substances into the kidneys of anaesthetized animals in physiological experiments, and for sundry other purposes requiring a known injection pressure in conjunction with some safety device to ensure against distortion of vessels from their normal paths by sudden raising of the pressure.

It is strongly recommended that as much as possible of the conducting part of the apparatus be made in one piece of glass as this eliminates several likely sources of air leakage, such as from rubber tubing joints on T-pieces, etc. For the same reason we prefer to use a Buckner funnel as a container in such injections as require only a litre or less of volume.

SUMMARY

The replacement in the compressed air type injection apparatus of the usual pressure stabilizing bottle by an open mercury reservoir into which dips a tube connected with the circuit conducting the main flow of compressed air combines, it is claimed, a constant pressure with an automatic maximum pressure safety valve.

REFERENCES

Narat, J. K., Loef, J. A. & Narat, M. (1936). On the preparation of multicoloured corrosion specimens. *Anat. Rec.* **64**, 155–160.

Tobin, C. E. (1947). A radioopaque-plastic injection mass. *Anat. Rec.* **98**, 137–145.

Trueta, J., Barclay, A. E., Daniel, P. M., Franklin, K. J. & Prichard, M. M. L. (1947). *Studies on the Renal Circulation*. Oxford: Blackwell Scientific Publications.

THE USE OF SAFRANIN-O FOR THE DEMONSTRATION OF CYTOPLASMIC NUCLEOPROTEINS

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The experiments to be described were originally designed to discover whether histochemical evidence for the presence of proteolytic enzymes or their precursors could be obtained. For this reason, attention was initially concentrated upon the zymogen granules of the pancreas and the pepsinogen granules of the peptic cells of the stomach. The animals used were rats and guinea-pigs.

The technique employed was based upon the fact that safranin and certain other related azine dyestuffs combine, apparently specifically, *in vitro*, with proteolytic enzymes. The first observations were made by Robertson (1907), who showed that when a drop of saturated Grübler's safranin was added to a solution of trypsin, a precipitate was formed. The observations were further extended by Holzberg (1913), who showed that the precipitate formed with trypsin possessed proteolytic activity. Marston (1923) demonstrated that the precipitate formed with a crude pancreatic extract possessed proteolytic, but neither lipolytic nor diastatic activity; he was also able to prepare trypsin, erepsin, pepsin and papain azine precipitates with safranin and other azine bases, such as neutral violet and neutral red. Marston suggested that the enzyme became linked to the dye through the basic nitrogen of the azine ring.

On the evidence from the *in vitro* experiments just mentioned, it was hoped that the perfusion of freshly killed animals with a safranin solution might result in the dye being 'fixed' in cells or regions of cells containing proteolytic enzyme systems.

It will be seen from what follows, that the expected results were not obtained, but that the dye was found to be firmly held only in cells the cytoplasm of which is known to contain a high concentration of ribose-nucleoprotein.

MATERIALS AND METHODS

The fourteen guinea-pigs and eight rats used in these experiments were killed with chloroform or ether. The thoracic aorta was exposed and cannulated, the right auricle punctured and the vascular system perfused with normal saline at 37°C. for 10 min. The saline was immediately followed by a 1% solution of filtered Grübler's safranin in normal saline at the same temperature. [In later experiments this safranin had to be replaced by one of another manufacture and this was found to be less reliable.] The perfusion with the dye was carried out more slowly and lasted up to 20 min. and, on an average, 50 ml. of the solution were used. After perfusion, pieces of stomach, pancreas, kidney and, in later experiments, liver and pituitary gland were removed. All these tissues were fixed in 10% formalin, but some pieces of stomach, pancreas and kidney were fixed in absolute alcohol or Bouin's fluid. The tissues were washed overnight in water, dehydrated through the alcohols, paraffin embedded and cut at 10μ.

Some sections were examined without further treatment, others were lightly counterstained with light green, and the nuclei stained with a 1 % aqueous solution of methyl green. Since acidified alcohol removes basic dyes from tissues, the effect of a 1 % HCl solution in 70 % alcohol upon the retention of the dye in the cells was also investigated.

In addition, one rat was perfused with a 1 % solution of neutral red in saline and another with a 1 % solution of neutral violet in saline. Also, a rat and a guinea-pig were perfused with a 1 % solution of methyl green in saline and one guinea-pig with a 0·5 % solution of toluidin blue in saline. These last two dyes were used as controls, since they are both basic substances but are chemically dissimilar to the azine dyes. Finally, small pieces of stomach and pancreas from an unperfused guinea-pig were fixed in 10 % formalin washed in water, soaked in 1 % safranin solution in saline overnight, dehydrated, embedded and cut. This was done to discover whether safranin was retained in any particular cells *after* fixation.

In some later experiments, when it appeared that the safranin retention in cells was associated with the presence of nucleoproteins rather than proteolytic enzymes, it was decided to investigate the possible 'fixation' of the dye by the Nissl substance of neurones. Difficulty, however, was encountered in getting satisfactory results after perfusion of the nervous system. In consequence, a different technique was used. Pieces of medulla were removed from guinea-pigs killed with ether and 15μ sections were immediately cut on the freezing microtome. These sections were placed for 30 min. in warm 1 % safranin in normal saline, after which they were rinsed in water and fixed in 10 % formalin overnight. The sections, after washing in water, were stained with light green in clove oil and mounted in balsam. Where the general cytoplasmic staining was strong, the dye was removed by treating the sections for 5 min. with acid alcohol before counterstaining.

RESULTS

Of the total of twenty-two perfusions carried out, those on three rats and five guinea-pigs were unsatisfactory. This was due either to a failure of the perfusion before the safranin was passed through, or to an incomplete perfusion of the dye.

Before the results are described in detail, it must be emphasized that these experiments are not considered to be comparable with those in which a dilute solution of a relatively non-toxic dye is applied to tissues intra- or supra-vitally. In such cases the cells may be regarded as being in a practically normal functional state. In these experiments, however, where the animals were first killed by a lipoid solvent anaesthetic and where, on an average, half an hour elapsed before the perfusion was complete, it seems almost certain that some post-mortem changes would have occurred within the cells, particularly in their membrane permeability. On the other hand, there was no evidence to suggest that the post-mortem changes were such as to result in any gross displacement or alteration of intracellular structures such as secretion granules or chromidial substance.

The effect of the method of fixation on the retention of the dye

Perfused pieces of stomach and pancreas from one rat were fixed in absolute alcohol and from one rat and one guinea-pig in Bouin's fluid. In neither case was

there any fixation of the dye, which was lost from the tissues during the fixation process itself. Both of these fixatives are protein precipitants and therefore act in a different manner from formalin, after the use of which the dye was retained in certain cells to be described below.

The retention of safranin in the cytoplasm of certain cells after perfusion

Immediately after a satisfactory perfusion the whole tissue was a brilliant reddish purple colour, but during the course of fixation, washing and dehydration the dye largely diffused out. After cutting and the removal of paraffin from the sections, there was generally some diffuse staining of the section as a whole in addition to the more intense staining of those cells which appeared to have an unusual affinity for the dye. The diffuse staining generally disappeared during downgrading through the alcohols, prior to staining the nuclei with methyl green. Alternatively, the diffuse stain could readily be removed by treatment with acid alcohol for a minute or two, but such treatment did not affect the dye in the specifically stained cells.

The retention of safranin in gastric and pancreatic cells

The results obtained were rather unexpected, both in rats and guinea-pigs. It was anticipated that if there was to be any fixation of the dye in these particular organs its attachment would most likely be to the proteolytic enzyme precursors, i.e. the pepsinogen granules of the stomach and to the zymogen granules of the pancreas. In no instances, however, were these granules coloured by the safranin, although certain regions of the cytoplasm of both peptic cells and the alveolar cells of the pancreas were seen to retain the dye. The retention of the dye in these cytoplasmic regions was, moreover, quite strong, since in well-perfused specimens treatment for 12 hr. in 1 % HCl in 70 % alcohol did not completely remove the safranin from them, although downgrading through the alcohols, or a brief rinse in acid alcohol was sufficient to remove the dye from the rest of the section. One exception to this last statement was observed in the stomach, where the cytoplasmic granules of certain, as yet unidentified, connective tissue cells showed a strong affinity for the dye.

The distribution and morphology of the safraninophil material in the cytoplasm of the alveolar cells of the pancreas was particularly characteristic. It recalled the basophil material known variously as the chromidial substance or ergastoplasm. This material, as is well known, occurs in the form of filaments and granules in the proximal part of the cytoplasm, where the mitochondria are primarily concentrated. The correspondence (Pl. 1, figs. 3, 4; Pl. 2, fig. 10) between the chromidial substance as revealed by basic dyes in routine preparations *after* normal fixation, and the strands and granules coloured by the safranin when applied *before* fixation was so close, that there seemed little doubt that the structures so stained were identical. The secretion granules were uncoloured.

Again, in the peptic cells, the safranin-stained material was in the form of threads and granules (Pl. 1, fig. 1; Pl. 2, figs. 6–9) strongly reminiscent of the ergastoplasm. It was possible to stain the cells by Heidenhain's iron-haematoxylin method—a procedure which demonstrated the pepsinogen granules in the distal part of the cell and temporarily obscured the 'safraninophil' material in other parts of the

cytoplasm; on destaining, however, with iron alum, the red ergastoplasmic material reappeared. Thus once more, there seemed little doubt that the cytoplasmic parts stained corresponded closely with the basophil material known to occur in peptic cells.

Recent studies using the ultra-violet light absorption technique (Caspersson, 1940), and the method of digestion with solutions of purified ribonuclease (Brachet, 1941) have produced powerful circumstantial evidence for the presence of a high concentration of ribose-nucleoproteins in these basophil ergastoplasmic regions of pancreatic and peptic cells (see Caspersson, 1947, pl. 2, fig. 2) and, indeed, for a large number of other cells known to have a high rate of protein synthesis (Caspersson, 1947; Thorell, 1947). In view of these findings, it was decided to investigate the fixation of safranin in certain other organs, the cells of which are known to possess cytoplasmic ribose-nucleoproteins. Those chosen were liver, pituitary and brain, and the results obtained are described below.

The retention of safranin in the liver, pituitary and brain

The highest concentration of safranin in the liver was in the cells at the periphery of the lobules close to the portal canals. The safraninophil material was either in the form of aggregates of small deeply staining granules distributed at random in the cytoplasm or the whole of the cytoplasm was stained, but to a lesser degree (Pl. 2, fig. 5). The former appearance resembled most closely the basophil material described in liver cells.

Although only one satisfactory perfusion of the guinea-pig anterior pituitary was obtained, the results are reported here. In sections of this specimen it was not possible to distinguish the cell types with certainty, but strands and granules of safranin-staining material were observed in what appeared to be basophil and acidophil cells. This was similar to the basophil material demonstrable in these cells after fixation in Helly's fluid, and staining with methylene blue-eosin mixtures.

As has been pointed out above, it was found necessary to alter the technique for the study of the neurones of the central nervous system. The essential condition of the other experiments was, however, maintained in that the tissue was subjected to the dye *before* fixation. Examination of the sections showed unquestionably, that the safranin had become 'fixed' to the Nissl substance (Pl. 1, fig. 2), and this fixation was resistant to several hours' treatment with acid alcohol. Furthermore, an examination of Auerbach's plexus in the safranin perfused stomach showed neurones with their Nissl substance dyed by the safranin.

The retention of safranin in the kidney

The kidney was initially studied together with the stomach and pancreas, in order to see whether the use of safranin might be a method for the histological demonstration of the proteolytic enzyme renin. Examination of the sections showed that the dye was held in certain tubules of the cortex, the medulla being very much less deeply stained. After treatment with acid alcohol, the dye was still found to be held in the cells of the proximal, and possibly in those of the distal convoluted tubules; the staining reaction was diffuse and the dye did not appear to be attached to any threads or particles, as was the case with peptic and pancreatic cells. In some

instances, the dye appeared to be adsorbed to the capillary walls of the glomeruli and to material precipitated in the cavity of Bowman's capsule.

The effect of staining with safranin after fixation

The technique used has already been described. It was found that the dye was very rapidly extracted during the embedding process from the pieces of guinea-pig's stomach. The examination of sections, prior to downgrading through the alcohols, showed a coloration of all cells and nuclei, as well as a strong staining, this time of the pepsinogen granules. Alcohol and acid alcohol were found to remove the safranin from the cells and pepsinogen granules with great rapidity—a marked contrast to what was observed in the previous experiments.

The retention of neutral violet and neutral red

The fundus of the rat's stomach was used to discover whether these two dyes, chemically related to safranin, were likewise fixed in the peptic cells. In both cases there was a retention of the dye in these cells. The rather pale colour of the neutral violet made it difficult to determine the exact nature of the cytoplasmic parts stained, but there was certainly no evidence that the pepsinogen granules took up the dye. The reaction with neutral red was diffuse, but there was no staining of pepsinogen granules.

The effect of perfusion with methyl green and toluidin blue prior to fixation

These substances were chosen as examples of powerful basic dyes, chemically dissimilar to the safranins. It was thought that if the retention of the basic dye, safranin, in certain cells was merely due to a combination with an acid component in the cytoplasm, then other basic dyes might act in a similar manner. This was not the case, however, with the two dyes used; they were rapidly washed out during embedding and downgrading through the alcohols and were quickly and completely removed by acid alcohol.

The retention of safranin by nuclei

The results here were variable. In a number of instances, and particularly in nerve cells, it was noted that the plasmosomes and more irregular granules, interpreted as karyosomes, held the safranin strongly. The dye could often be masked with a nuclear stain such as methyl green, but the red colour reappeared after the removal of the green dye with acid alcohol.

DISCUSSION

From the results described above, the following principal points emerged:

(1) The safranin was firmly held, even after acid alcohol treatment, by the cytoplasm of certain cells, when the subsequent fixation of the specimen was in formalin. It was not held when subsequent fixation was in alcohol or Bouin's fluid. Of these three fixatives, only formalin is a non-precipitant of proteins.

(2) The application of the dye *after* fixation in formalin, did not result in its retention by any cells in the tissues studied. In fact, in the stomach there was

a qualitative difference in that the pepsinogen granules were temporarily stained, whereas in safranin perfusion *before* fixation they were never coloured.

(3) It did not seem likely that the holding of the dye was directly related to its basic nature, since the two chemically dissimilar basic dyes used—toluidin blue and methyl green—were rapidly removed, both during the embedding process and afterwards.

(4) The chemically related dyes, neutral violet and neutral red, were also found to be held in cells similar to those which react strongly with safranin. The staining, however, particularly with neutral red, was more diffuse and its precise intracellular distribution was difficult to determine.

Recent investigations by Schultz & Caspersson (1940), Caspersson (1947), Thorell (1947), and others, have provided evidence which suggests that the nucleoproteins of the cytoplasm play a very important part in intracellular protein synthesis. A high concentration of ribose-nucleoproteins appears to be associated, for example, with embryonic cells, developing blood cells and various gland cells possessing a high protein turnover. Moreover, it seems more than probable, that in various glandular cells of an exocrine type, the nucleoproteins are largely concentrated in the strongly basophil chromidial substance or ergastoplasm. This certainly seems to be true for pancreatic and peptic cells. The basophil material present in the chromophil cells of the pituitary and in liver cells, as well as the Nissl substance of nerve cells (Hyden, 1947), also contains a high proportion of ribose-nucleoproteins. In all these instances it is possible to destroy the characteristic basophilia by the incubation of tissue sections with the enzyme ribonuclease for which ribose-nucleoprotein is believed to be a specific substrate. (Brachet, 1941, 1947; Dempsey & Wislocki, 1945; Davidson & Waymouth, 1943; Gersh & Bodian, 1943).

The close correspondence between the morphology and position of the cytoplasmic structures 'fixing' safranin in these experiments and the ergastoplasm and Nissl substance suggests that it is the nucleoproteins of the cell which are binding the dye. It is not necessarily true, however, that the dye combines specifically with ribose-nucleoproteins, since, as has been mentioned, the karyosomes (which contain desoxyribose-nucleoprotein) often showed a strong affinity for the dye. In nerve cells, the plasmosomes which contain ribose-nucleoprotein were always strongly reacting.

It may be relevant that Dustin (1947) has suggested that certain vital dyes, such as neutral red, which accumulate in pre-existing and newly formed intracellular vacuoles (such as those of the Golgi zone of various cells), do so because they become attached to the acidic nucleoprotein molecules which he believes accumulate in these structures. There appears to be no suggestion, however, of any specific chemical attachment. Of more significance is the observation communicated to me by Prof. Frank Dickens, F.R.S., to the effect that nucleic acids combine, *in vitro*, with phenosafranin (the substance of which safranin-*O* is a derivative) to form a precipitate.

In an attempt to account for the observations which have been made, the two following alternative hypotheses are put forward. First, the nucleic acid part of the nucleoprotein molecule may combine with the safranin to give a fairly stable compound, which is not destroyed by formalin and which is resistant to subsequent treatment with acid alcohol. Secondly, if, as has been recently suggested by

Brachet (1947), the nucleic acids of the cytoplasm are associated with proteolytic and other enzyme systems, then the safranin may combine with these associated proteolytic enzymes, presumably by the same mechanism as operates *in vitro* (see Introduction). On this hypothesis, the failure of the dye to combine with pepsinogen or zymogen granules must be assumed to be due to the fact that these bodies contain enzyme precursors and not the active enzymes themselves, and in consequence behave differently.

The results with kidney tissue are anomalous according to either hypothesis, since there is little ribose-nucleoprotein in kidney cells. These cells are, however, unique in respect of their absorbing and secretory properties, and the diffuse retention of the dye by proximal convoluted tubules might be a special case connected with these properties; but since such an explanation is dependent upon the assumption that the kidney cells in these experiments were sufficiently undamaged to function normally, it seems an unlikely one.

SUMMARY

1. Safranin and certain other azine dyes were found to be firmly held in the acinar cells of the pancreas, peptic cells of the stomach, liver cells, nerve cells of Auerbach's plexus and the chromophil cells of the anterior pituitary, following post-mortem perfusion with the dyes, and subsequent fixation of the tissues in formalin. The dye was also retained in nerve cells of the brain after the special procedure already described.

2. The intracellular distribution of the dyes and the distribution of the cytoplasmic nucleoproteins were found to be in close correspondence.

3. It is suggested that the dye combines directly, either with the nucleic acids, or else with proteolytic enzymes which may be associated with them.

I would like to thank Prof. F. Goldby of the Anatomy Department, St Mary's Hospital Medical School, for his interest and helpful criticism.

REFERENCES

Brachet, J. (1941). La localisation des acides pentosenucléiques dans les tissus animaux et les oeufs d'Amphibiens en voie de développement. *Arch. Biol., Paris*, **53**, 207–257.

Brachet, J. (1947). Nucleic acids in the cell and embryo. In 'Nucleic Acid'. *Symposia Soc. exp. Biol.* no. 1, pp. 207–224.

Caspersson, T. (1940). Methods for the determination of the absorption spectra of cell structures. *J. R. micr. Soc.* **60**, 8–15.

Caspersson, T. (1947). The relations between nucleic acid and protein synthesis. In *Symposia Soc. exp. Biol.* no. 1, pp. 127–151.

Davidson, J. N. & Waymouth, C. (1943). The histochemical demonstration of ribonucleic acid in mammalian liver. *Proc. roy. Soc. Edinb.* B, **62**, 96–98.

Dempsey, E. W. & Wislocki, G. B. (1945). Histochemical reactions associated with basophilia and acidophilia in the placenta and pituitary gland. *Amer. J. Anat.* **76**, 277–295.

Dustin, P. Jnr. (1947). Ribonucleic acid and the vital staining of cytoplasmic vacuoles. In 'Nucleic Acid'. *Symposia Soc. exp. Biol.* no. 1, pp. 114–126.

Gersh, I. & Bodian, D. (1943). Histochemical analysis of changes in rhesus motoneurons after root section. *Biol. Symp.* **10**, 163–184.

Holzberg, H. L. (1913). A new method of isolating trypsin. *J. biol. Chem.* **14**, 335–339.

HYDEN, H. (1947). Protein and nucleotide metabolism in the nerve cell under different functional conditions. In 'Nucleic Acid'. *Symposia Soc. exp. Biol.* no. 1, pp. 152–162.

MARSTON, H. R. (1923). The azine and azonium compounds of the proteolytic enzymes. I. *Biochem. J.* **17**, 851–859.

ROBERTSON, T. B. (1907). Studies in the chemistry of the iod-protein compounds. IV. *J. biol. Chem.* **2**, 317–383 (p. 343).

SCHULTZ, J. & CASPERSSON, T. (1940). Ribonucleic acids in both nucleus and cytoplasm, and the function of the nucleolus. *Proc. nat. Acad. Sci., Wash.*, **26**, 507–515.

THORELL, B. (1947). Studies on the formation of cellular substances during blood cell production. *Acta med. scand.* **129**, *Suppl.* 200, 1–120.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. Guinea-pig stomach. Peptic cells (*p*); oxyntic cells (*o*). Safranin perfusion, nuclei stained with methyl green. ×1000.

Fig. 2. Nerve cells from medulla of guinea-pig, showing retention of safranin in the Nissl substance. Stained with safranin prior to fixation (see text), and counterstained with light green. ×500.

Fig. 3. Pancreas of dog, showing distribution of chromidial substance (*c*), for comparison with fig. 4. Zenker-formol fixation, Ehrlich's haematoxylin and eosin. ×400.

Fig. 4. Guinea-pig pancreas, showing retention of safranin in the basal regions of the cells, where chromidial substance is normally present (*c*). Safranin perfusion; nuclei stained with methyl green. ×600.

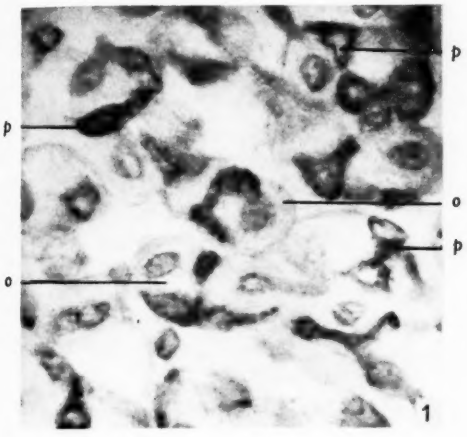
PLATE 2

Figs. 5–10. Camera-lucida drawings of safranin perfused guinea-pig material.

Fig. 5. Liver cell showing distribution of safraninophil material.

Figs. 6–9. Cells from fundus of stomach showing the distribution of the safraninophil material which is in the form of threads and granules in the peptic cells (*p*). Note that this material is absent from the oxyntic cells (*o*).

Fig. 10. Section of pancreatic alveolus. Notice that the distribution of the safraninophil material corresponds with that of the chromidial substance (cf. Pl. 1, fig. 4). *z* = region of unstained zymogen granules.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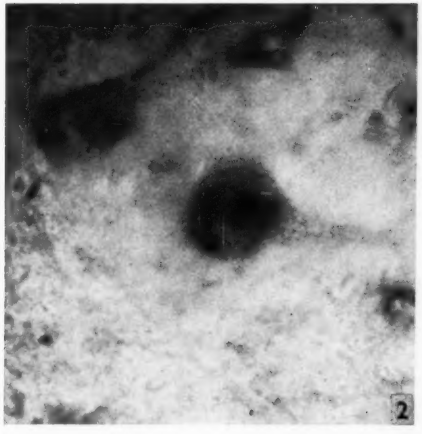
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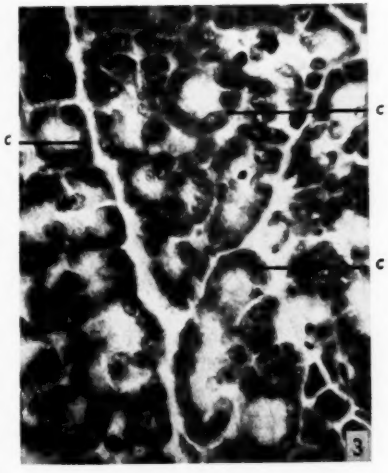
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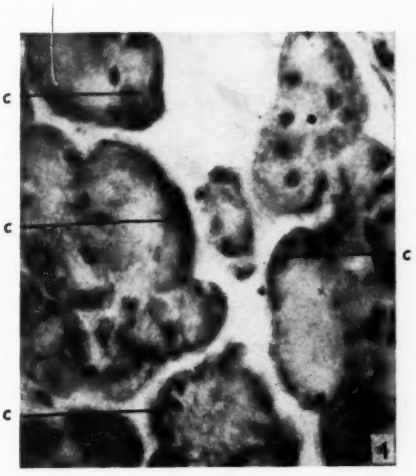
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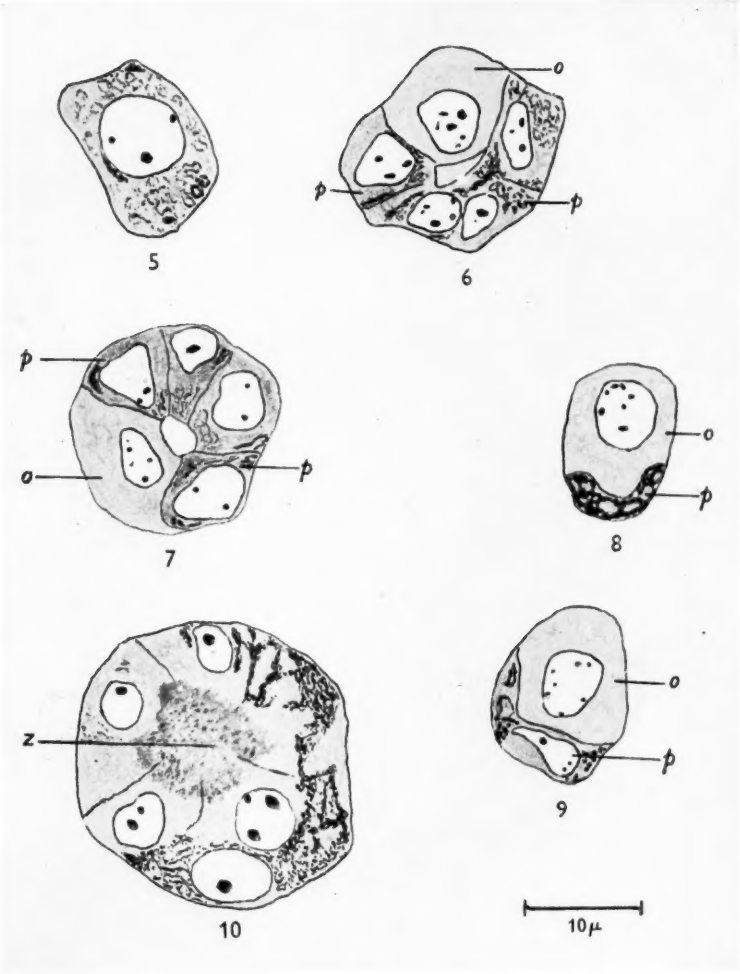
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DENDRITIC CELLS IN PIGMENTED HUMAN SKIN

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INTRODUCTION

It has long been known that the characteristic coloration of the skin of the pigmented human races is due to the presence of the pigment melanin. This occurs in the epidermis where it is most abundant at the level of the deepest or basal layer of cells. As to the source of origin of this pigment and the intimately connected problem of the exact cellular composition of the tissue that contains it, complete unanimity of opinion has not yet been reached, some authors still maintaining that the Malpighian cells are the site of melanogenesis (see reviews by Hoepke, 1927; Bloch, 1929; Percival & Stewart, 1930; Laterjet, 1938; Meirowsky, 1940; Masson, 1948).

In guinea-pig epidermis, a compound tissue in whose structure two anatomically distinct types of cell participate—Malpighian cells with their specialized derivatives, and dendritic cells—melanogenesis has recently been shown to be an exclusive property of the latter (Billingham, 1948). Two distinct 'true breeding' races of these branched cells exist: the 'pigmented' dendritic cells which occur in the black epidermis of the spotted black-and-white guinea-pig and which are responsible for its pigment formation, and the anatomically identical 'white' dendritic cells which are found exclusively in the white epidermal regions. 'Pigmented' and 'white' dendritic cells differ only in that the former are endowed with the power of melanogenesis. The epidermis of ordinary white human skin has a similar twofold composition, but so far as pigmentary activity is concerned it resembles the skin of the so-called albino guinea-pig in that it has a latent capacity for pigment formation. In this animal the epidermis is normally white, probably because of the presence of an inhibitor (Onslow, 1915; Ginsburg, 1944), but it may be caused to blacken by such a mild stimulus as cold weather. It may be added that in the white skin of the spotted guinea-pig no merely physical stimulus is known which will initiate even the slightest degree of melanogenesis (Lewin & Peck, 1941; Ginsburg, 1944).

In view of these considerations there seemed to be the strongest indirect evidence that the epidermis of pigmented human skin would have a functional and anatomical dendritic cell system closely resembling that present in pigmented guinea-pig skin. The object of this study has been to obtain direct evidence in support of this analogy.

In this paper the term 'pigmented human skin' refers only to that of Indian or Negro and no attempt has been made to differentiate between the two types. The two main techniques used, although briefly described in a previous paper (Billingham, 1948), are here reported in some detail, in the hope that they will be of use to other students of cutaneous pigmentation.

MATERIAL AND METHODS

Pieces of normal pigmented skin (made available through the kindness of Prof. T. Pomfret Kilner, Dr H. M. Hanschell and Mr Dallas Ross) from various sites on the bodies of Indians and Negroes have been used in this study. The bulk of the material

consisted of trimmings from Thiersch grafts (i.e. very thin sheets of skin comprising the epidermis and only the superficial part of the dermis).

Sections. The fresh material was fixed in formol-mercuric chloride, dehydrated in an ethyl alcohol series and, after the use of cedarwood oil followed by ligroin as antemedia, the tissue was embedded in paraffin wax. Sections were cut at 8–10 μ and were lightly stained with Mayer's carmalum or Ehrlich's haematoxylin and eosin.

Whole mounts of 'Split' skin. The skin-splitting technique adopted (Medawar, 1941) depends upon the enzymic dissolution of the fine elastic fibres that unite the epidermis to the dermis. Freshly obtained Thiersch graft shavings which had previously been vaselined on their cuticular surface were cut into fragments of approximately 0·5 cm.^{2} These were floated on to a Seitz-filtered 0·5% solution of commercial trypsin powder in Ringer-bicarbonate containing 1:100,000 phenol red and adjusted thereby to pH 7·8, and digested for about 30 min., or longer if necessary (depending on the thickness of the material), at 38° C. The fragments were then rinsed in unbuffered Ringer's solution, blotted free from excess fluid, and then carefully flattened out on a dry slide, cuticular surface lowermost. The thin layer of dermal tissue was then lifted off with fine forceps leaving the epidermis behind as a thin intact sheet. The sheets of 'pure' epidermis so obtained (see Pl. 1, figs. 3–5) and referred to hereafter as 'split' skin were either fixed directly in formal-calcium (Baker, 1944), dehydrated in an ethyl alcohol series, cleared in clove oil and mounted in balsam, or immediately after fixation were treated with the 'Dopa' reagent.

The 'Dopa' reaction. (See Bloch, 1929.) So far as the epidermis is concerned the Dopa reaction affords a perfectly valid method of selectively revealing those cells which possess an active melanogenic system or enzyme complex (Russell, 1939; Ginsburg, 1944). It is based upon the fact that when fresh or freshly formal-fixed epidermal tissue is placed in a suitably buffered solution of *l*-3,4-dihydroxyphenylalanine (Dopa), a likely precursor of melanin (Raper, 1927), certain cells are able to bring about its intracellular oxidation to Dopa-melanin, becoming intensely blackened in the process; i.e. they are Dopa-positive.

Sheets of pure epidermis immediately after splitting were fixed for about half an hour in formal-calcium, rinsed in distilled water and transferred to the Dopa substrate freshly prepared by adding 2 ml. of Sörensen M/15 potassium dihydrogen phosphate solution (KH_2PO_4) and 8 ml. of Sörensen M/15 disodium phosphate solution (Na_2HPO_4) to 25 ml. of the stock 1:1000 Dopa solution in distilled water. The tissue, placed in an open vessel to allow free access of air, was incubated in this substrate at 38° C. for half an hour, after which the substrate was replaced by a freshly made-up solution and the incubation was continued for a further 2½–3 hr. The reaction was at an end when the solution had taken on a sepia-brown coloration. After rinsing in distilled water the standard fixation and mounting procedure was carried out.

OBSERVATIONS AND CONCLUSIONS

Study of transverse sections of full thickness pigmented skin, irrespective of the site on the body from which it was derived, shows that although melanin granules occur at all levels throughout the epidermis, and in small quantities in the superficial dermis, it is always at about the level of the deepest Malpighian cells that the pigment

Tabular summary of the epidermal glial system in man and guinea-pig

Type of cell	Occurrence	Normal 'colorimetric' description	Pigmentary activity	Notes
Pigmentary dendritic cell	Coloured human skin, e.g. Indian or Negro	Pigmented or black dendritic cell	Has a high level of melanogenesis which is intrinsically maintained	Cannot be distinguished in unstained or un-Dopa'd preparations because melanin granules are golden-brown in colour and relatively large. Dopa-reaction is essential
	Black guinea-pig skin	Ditto	Ditto	Easily visible in unstained 'split' skin preparations (Billingham, 1948) since melanin granules are black and very minute in size
	White human skin	'White' or non-pigmented dendritic cell	Normally very slight or absent	Possesses necessary enzyme system for pigment formation but an inhibitor is present (Rothman *et al.* 1946). Suitable stimuli, e.g. ultra-violet light, will initiate limited degree of melanogenesis (e.g. sun-tan) probably because of destruction or removal of 'inhibitor'
	White skin of albino guinea-pig	Ditto	Can be evoked by appropriate stimuli	Can be caused to blacken, e.g. after exposure to cold. Dendritic cells can then be seen in 'split' skin preparations (Billingham & Medawar, 1948). Possess necessary enzyme system for melanogenesis but an inhibitor normally present. (Onslow, 1915; Ginsburg, 1944)
Non-pigmentary dendritic cell	White skin of spotted black-and-white guinea-pig ? wherever there is 'recessive' spotting in rodents	'White' or non-pigmented dendritic cell	Absent and cannot be evoked	No form of physical stimulus known which will initiate any trace of pigmentation (Lewin & Peck, 1941). *Do not* possess necessary enzyme system for pigment formation

Synonyms: melanophore, melanoblast, chromatophore, stellate cell, clear cell, 'cellule amboceptrice'.

is found at its greatest concentration (Pl. 1, figs. 1, 3; Pl. 2, figs. 9, 10). Here it may be so abundant as to obscure the boundaries of the cells (Pl. 2, figs. 9, 10). The pigment granules are spherical, uniform in size and of a deep golden brown colour in transmitted light, so differing markedly from those of black guinea-pig epidermis, which are black. The capping and partial enveloping of the basal-layer cells by dense masses of these pigment granules is as prominent here (Pl. 1, fig. 3; Pl. 2, fig. 9) as in the guinea-pig. In such preparations absolutely no indication of the presence of dendritic cells can be found.

In sections of 'split' skin, in the preparation of which the tryptic digestion process had been prolonged to the point at which there was a tendency for individual epidermal cells to break away from the epidermal sheet, dendritic cell processes can often be seen still applied to it and apparently anchoring isolated basal-layer cells to the sheet (Pl. 1, fig. 6; Pl. 2, figs. 10, 11).

In unstained whole mounts of 'split' skin, examined with their cuticular surfaces lowermost, careful search rarely if ever reveals a trace of a dendritic cell (see Pl. 1, fig. 4). This is in contrast to similar preparations of guinea-pig skin in which the pigmented dendritic cells stand out boldly by virtue of their content of opaque melanin granules which also enable the processes arising from their perikarya to be followed out to their finest terminal twigs.

The Dopa-treated 'split' skin preparations have been the mainstay of this study. Examination of these reveals that dendritic cells are present in exactly the same position and abundance as are their 'white' homologues in white human skin from corresponding regions. Their perikarya occur at about the same level as the basal-layer cells (Pl. 1, figs. 2, 7, 8) and, as a single layer of discretely scattered cells among the latter, they follow faithfully the complex 'hill and valley' relief pattern which the lower surface of the separated epidermal sheet presents (Pl. 1, figs. 4, 5; Pl. 2, fig. 12). In this pattern the 'hills' are the downward prolongations of the epidermis into the dermis while the 'valleys' are really the spaces which were formerly occupied by the upward projection of the dermal papillae which, especially in thigh skin, tend to have flattened tops above which the epidermis is at its minimum thickness. The dendritic cells are found in highest concentration along the summits and sides of the downwardly directed epidermal ridges, while along the 'valleys' corresponding to the shallowest and thinnest regions of the epidermis they are more sparsely distributed. In these latter regions the branching systems arising from the individual dendritic cells tend to ramify more in the horizontal plane, thus enabling their relationships to neighbouring Malpighian cells and to cells of their own type to be studied (Pl. 2, figs. 12, 14). These preparations also reveal that a proportional relationship exists between the degree of epidermal pigmentation and abundance of dendritic cells.

The epidermis of pigmented human skin is thus identical in its anatomical constitution to nonpigmented epidermis (see Pl. 2, fig. 13), being composed of two types of cell: Malpighian cells and dendritic cells. Functionally, of course, the dendritic cells in pigmented skin differ from those in white skin in that they maintain in the epidermis a constant and relatively high concentration of pigment, while the nonpigmented dendritic cells in white skin can only effect a perceptible pigmentation of the epidermis as a result of some form of external stimulus, e.g. ultraviolet light, irritants, etc. This pigmentation never reaches a very high level of concentration and for its indefinite duration is dependent upon the continued application of the external stimulus. Rothman, Krysa & Smiljanic (1946) have suggested that white human epidermis contains a factor, probably sulphydryl compounds, with an inhibitory action on melanogenesis. On the basis of this evidence they have suggested that in melanoblasts (dendritic cells) both substrate and active enzyme are present but no reaction takes place between them because of the presence of the inhibitor. Melanogenic stimuli act by oxidizing or otherwise destroying the inhibitor, thus allowing

melanogenesis to take place. This would account for the similarity, with respect to pigmentary activity, between white human skin and that of the albino guinea-pig which has previously been referred to.

These pigmented dendritic cells in human material are very similar both anatomically and functionally to the dendritic cells found in the black skin of the guinea-pig. They differ from the latter in that they cannot normally be distinguished by virtue of their own melanin content, either in sections or in 'split' skin preparations; the use of the Dopa reaction, which is a sensitive colorimetric test for their melanogenic system, is essential. This difference is probably due in large part to the intense black colour and fine grain of guinea-pig melanin compared with the golden-brown colour of the human pigment granules.

As in the case of the pigmented skin of the guinea-pig there is ample evidence that melanogenesis is an exclusive property of these pigmented dendritic cells and that the pigment granules present within the ordinary basal-layer or Malpighian cells, often in considerable numbers, are derived at second hand from the dendritic cells by a process which may almost be compared to injection. As Masson (1948) has pointed out, these cells are both secretory and excretory in function and may therefore be considered as glandular cells. Unlike endocrine and exocrine glands, however, their product, melanin, is secreted into other cells across the terminal end-caps of the dendritic cell branches. Masson has called this 'cytocrine' activity.

In mammalian epidermis dendritic cells comprise a definite cell system as definite and specific in its own right as the reticulo-endothelial system. Billingham & Medawar (1948) have called it the *epidermal glial system*. To avoid any confusion which may have arisen with respect to terminology, a comparative summary of the 'epidermal glial system' in man and the guinea-pig has been included (see p. 111).

SUMMARY

1. It is shown that the pigmented epidermis of Indian and Negro skin is exactly similar in its anatomy to that of the white races, being composed of the more or less rounded Malpighian cells with their derivatives and the branched dendritic cells.

2. There is, however, a difference in functional activity between dendritic cells in the two types of epidermis, in that those present in pigmented tissue, like those of the black guinea-pig, have a high rate of melanogenesis which is intrinsically maintained, while those in white epidermis are capable only of a relatively slight degree of pigmentary activity as a consequence of the application of suitable external stimuli.

3. Attention is drawn to the glandular or 'cytocrine' role of pigmented dendritic cells.

The author wishes to express his sincere gratitude to Prof. P. B. Medawar who supervised the work recorded in this paper and gave great assistance at all stages, and to Miss Jean Morpeth who has given invaluable technical assistance.

REFERENCES

BAKER, J. R. (1944). The structure and chemical composition of the Golgi element. *Quart. J. micr. Sci.* **85**, 1–71.

BILLINGHAM, R. E. (1948). Dendritic cells. *J. Anat., Lond.*, **82**, 93–109.

BILLINGHAM, R. E. & MEDAWAR, P. B. (1948). Pigment spread and cell heredity in guinea-pig's skin. *Heredity*, **2**, 29–47.

BLOCH, B. (1929). The problem of pigment formation. *Amer. J. med. Sci.* **177**, 609–618.

GINSBURG, B. (1944). The effects of the major genes controlling coat colour in the guinea-pig on the Dopa oxidase activity of skin extracts. *Genetics*, **29**, 176–198.

HOEPKE, H. (1927). 'Die Haut.' In von Möllendorff's *Handbuch der mikroskopischen Anatomie des Menschen*, **3**, i, 1–116. Berlin: J. Springer.

LATERJET, R. (1938). La physiologie normale du pigment mélanique cutané chez l'homme. *Biol. méd.* **28**, 1–40.

LEWIN, M. L. & PECK, S. M. (1941). Pigment studies in skin grafts on experimental animals. *J. Invest. Derm.* **4**, 483–504.

MASSON, P. (1948). Pigment cells in man. *Spec. Publ. N.Y. Acad. Sci.* **4**, 15–51.

MEDAWAR, P. B. (1941). Sheets of pure epidermal epithelium from human skin. *Nature, Lond.*, **148**, 783.

MEIROWSKY, E. (1940). A critical review of pigment research in the last hundred years. *Brit. J. Derm.* **52**, 205–217.

ONSLOW, H. (1915). A contribution to our knowledge of the chemistry of coat-colour in animals and of dominant and recessive whiteness. *Proc. roy. Soc.* B, **89**, 36–58.

PERCIVAL, G. H. & STEWART, C. P. (1930). Melanogenesis: a review. *Edinb. med. J.* **37**, 497–523.

RAPER, H. S. (1927). The Tyrosinase-Tyrosine reaction. VI. Production from tyrosine of 5:6-dihydroxyindole and 5:6-dihydroxyindole-2-carboxylic acid—the precursors of melanin. *Biochem. J.* **21**, 89–96.

ROTHMAN, S., KRYSA, H. F. & SMILJANIC, A. M. (1946). Inhibitory action of human epidermis on melanin formation. *Proc. Soc. exp. Biol., N.Y.*, **62**, 208–209.

RUSSELL, W. L. (1939). Investigation of the physiological genetics of hair and skin colour in the guinea-pig by means of the Dopa reaction. *Genetics*, **24**, 645–667.

EXPLANATION OF PLATES

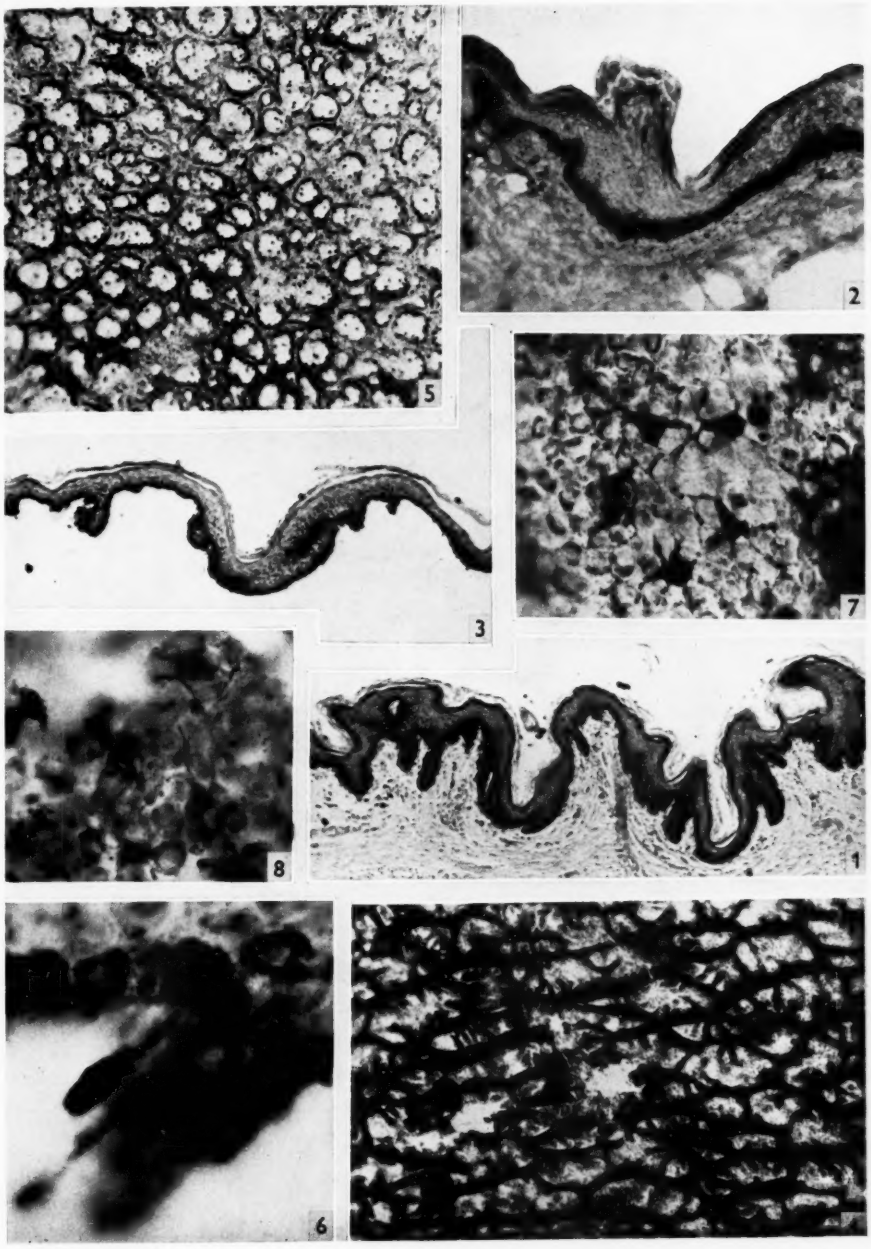
PLATE 1

Fig. 1. Vertical section through pigmented skin of Negro's scrotum. Observe the complex corrugation pattern characteristic of scrotal skin. The epidermal ridges penetrate rather deeply into the underlying dermis. Pigment density is greatest at the level of the lowermost cells of the epidermis. The dermis is almost devoid of melanin. Lightly stained with Ehrlich's haematoxylin and eosin. ×58.

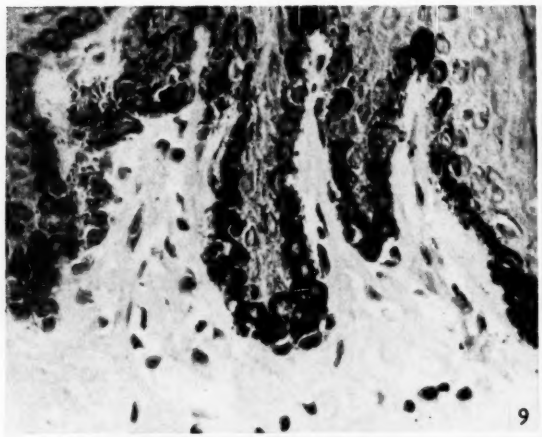
Fig. 2. Vertical section of pigmented scrotal skin of Negro cut at 40μ on the freezing microtome and treated with Dopa. The bodies of the dendritic cells and their processes can just be distinguished due to the intracellular formation of black Dopa-melanin. In the epidermis Dopa-positive cells are only found at the level of the Malpighian layer. ×75.

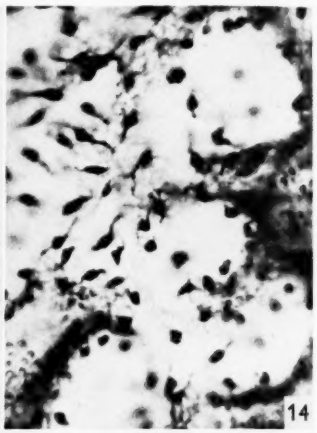
Fig. 3. Vertical section of 'split' pigmented scrotal epidermis. The epidermis has been separated from the dermis by tryptic digestion and the sections prepared from it lightly stained with Ehrlich's haematoxylin and eosin (compare with fig. 1). Although pigment is mainly restricted to the level of the basal-layer cells, many of the epidermal cells at higher levels are 'capped' with pigment in a highly characteristic manner. ×67.

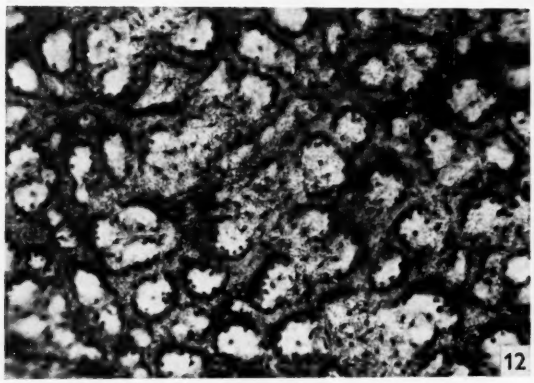
Fig. 4. Whole mount preparation of a sheet of pure pigmented epidermis ('split' skin) of Negro's scrotum, unstained and viewed from the underside. Note the complex 'hill and valley' contour or ridging pattern which the lower surface of the epidermis makes with the dermis. This is often characteristic and specific for the different types of epidermal tissue found throughout the body (compare with that of thigh skin, figs. 5 and 12). The 'hills' are the epidermal ridges while the 'valleys' are the spaces originally occupied by the dermal papillae. In this preparation no indications of dendritic cells can be distinguished. Pigment is mainly concentrated along the crests and sides of the epidermal ridges. ×27.

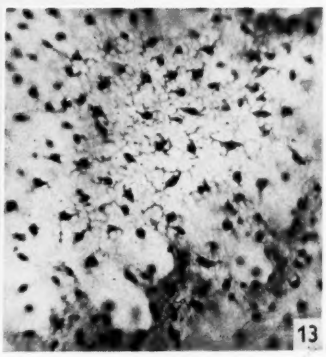


BILLINGHAM—DENDRITIC CELLS IN PIGMENTED HUMAN SKIN









BILLINGHAM—Dendritic cells in pigmented human skin

Fig. 5. Whole mount of 'split' thigh skin of Indian which has been treated with Dopa. The preparation is viewed from the underside. Observe and compare the ridging pattern with that of scrotal epidermis (fig. 4). The dendritic cell bodies have been 'stained' black by the Dopa and can be seen clearly. They are more closely distributed in the epidermal ridges which are also the regions of maximum depth of pigmentation. ×50.

Fig. 6. Vertical section of 'split' pigmented scrotal skin. One of the ordinary basal-layer cells has almost broken away from the epidermal sheet, being retained only by a process from a dendritic cell which ends upon it as an intimately-applied cap or end-button. Unstained. ×1080.

Figs. 7, 8. Pigmented dendritic cells in a sheet of pure epidermis from Negro's scrotum which has been 'stained' with Dopa. Their perikarya and the processes which arise from them are intensely blackened by Dopa melanin. Note the melanin granules in the cytoplasm of the Malpighian cells in fig. 8. ×383.

PLATE 2

Fig. 9. Vertical section of pigmented skin of negro's scrotum to show the highly characteristic 'capping' distribution of melanin in relation to the basal-layer cells of the epidermis. Lightly stained with Ehrlich's haematoxylin. ×383.

Figs. 10, 11. Vertical sections of 'split' pigmented scrotal skin lightly stained with Ehrlich's haematoxylin and eosin. The enzymic splitting process was deliberately prolonged to the point at which cells had begun to separate from the epidermal sheet. Dendritic cell processes can be seen still adherent to some of the Malpighian cells which are breaking away. Note also the almost complete localization of pigmentation to the level of the basal-layer cells of the epidermis. Fig. 10, ×383; fig. 11, ×383.

Figs. 12, 14. Pigmented dendritic cells in pure pigmented epidermis of Indian's thigh skin stained by the Dopa method. At the bottoms of the epidermal 'valleys' the dendritic cells, though more sparsely distributed, tend to have their branches spread out more in the horizontal plane. Fig. 12, ×80; fig. 14, ×120.

Fig. 13. White dendritic cells of 'split' white (Caucasian) human skin. The preparation has been stained supravitally with methylene blue solution in Ringer. Note that the distribution, size, number and mode of branching of the dendritic cells is just the same as with pigmented skin (compare with figs. 12 and 14). ×150.

OBSERVATIONS ON THE SNOUT OF *VARANUS*, AND A COMPARISON WITH THAT OF OTHER LIZARDS AND SNAKES

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INTRODUCTION

The family Varanidae comprises the single genus of monitor lizards which are widely distributed throughout the tropical and subtropical regions of the Old World. This genus includes the largest of all living lizards; the different species are conservatively modified in adaptation to terrestrial, partly arboreal and partly aquatic habits. *Varanus* occupies a somewhat isolated position among the living members of the order Sauria; it has generally been recognized as an ally of the extinct mosasaurs (Williston, 1925; Camp, 1923, 1940), and has also been regarded as related to the ancestors of snakes (Camp, 1923; Romer, 1945). In spite of the interest of the group, however, no systematic investigation of the chondrocranium of *Varanus* has yet been made, although several studies of the bones and other cranial structures are available. Kesteven (1940) has made some observations on the development of the skull in *V. varius*, but these are restricted to the base of the cranium. Pratt (1948) has described and figured the nasal and vomeronasal organs of *V. monitor* and *V. niloticus*; his account, however, is necessarily brief as it forms part of a general survey of the nasal region throughout the Sauria. It was therefore thought desirable to attempt a more detailed study of the anatomy of the snout in this genus, and to compare conditions present in *Varanus* with those in other lizards and snakes. Since no general description of the snout of any reptile is available, it was also hoped that this account might be of wider interest.

This investigation is principally based on serial sections through the nasal region of a juvenile example of each of the following species: *V. monitor* (Linn.), (*syn. V. bengalensis*), *V. salvator* (Laurenti) and *V. niloticus* (Linn.). I am much indebted to Mr C. W. M. Pratt for the loan of his sections of *V. niloticus*, and also for opportunities for discussing my results with him. The graphic reconstructions and most of the sections figured are based on the specimen of *V. monitor*, which was sectioned at 14 μ and stained with haematoxylin and eosin. This specimen measured approximately 240 mm. from snout to tail-tip (head-length 25 mm.) and was obtained near Meiktila in central Burma. Other *Varanus* material included the head of an adult specimen of *V. salvator*, a skull assigned to the same species, a juvenile skull of *V. monitor* and another of *V. niloticus*. I am indebted to Mr H. W. Parker and Dr Malcolm Smith of the British Museum of Natural History, and to Dr R. E. Rewell and the Zoological Society of London for the gift or loan of these latter specimens. My thanks are also due to Mr W. Warwick James for the young specimen of *Tupinambis teguixin* from which the sections shown in Text-fig. 11C and Pl. 1B were prepared.

TERMINOLOGY

In a monograph on the snout of Crossopterygian fishes and lower gnathostomes in general, Jarvik (1942) has pointed out the shortcomings of the nomenclature usually employed in describing the structures in this region. In particular, he draws attention to the fact that inadequate distinction is usually made between cavities and the structures, both skeletal and non-skeletal, which enclose them. Since Jarvik's terminology seems to be an advance on any previously used and may, with certain modifications, be applied to vertebrates in general, it has been employed as far as possible throughout this account.

The endocranial skeleton of the nasal region is termed the nasal capsule. Inside this lie the nasal sacs which can be subdivided into various regions, as described by Beecker (1903) and Pratt (1948). Each nasal sac communicates with the outside by means of an anterior nasal tube opening by an anterior external nostril, and with the mouth or pharynx by an internal nasal or choanal tube. The nasal and oro-pharyngeal openings of each choanal tube are termed the inner and outer choanae respectively, following the usage of Born (1879), who gave the first comprehensive description of this region in Sauria. Both anterior and internal nasal tubes pass through fenestrae, first in the endocranial and then in the dermal skeleton; the fenestrae for the anterior nasal tubes are termed the fenestra endonarina anterior and fenestra exonarina anterior respectively, while those for the choanal tubes are termed fenestra endochoanalis and fenestra exochoanalis respectively. The posterior nasal tubes and posterior external nostrils of fishes are regarded by Jarvik as homologous with the lachrymal ducts and puncta of tetrapods. In the case of Squamata the term lachrymal duct is preferable to the more familiar naso-lachrymal of mammalian anatomy, since in the former group its anterior opening is more closely related to the mouth than to the nasal sac in many forms.

Since the variations in the saurian palate, which have been almost entirely neglected in the English literature, appear of considerable importance in a general understanding of palatal morphology in amniotes, the terminology of this region has also received some attention. An attempt has been made to apply the principles of Jarvik's nomenclature to the palate, which may be defined as all those tissues which lie between the nasal sacs above, and the mouth or pharynx below. These tissues may be subdivided into:

(*a*) The floor of the nasal capsule, which is conveniently described by the usual terminology of the chondrocranium.

(*b*) The dermal skeleton, here called the bony palate.

(*c*) The soft tissues lying between the bony palate and the oral and pharyngeal cavities; the term superficial palate has been arbitrarily applied to these.

The duct of Jacobson's organ passes through these palatal tissues in all adult Squamata, and opens into the oral cavity, its epithelium becoming continuous with that of the superficial palate. The fenestrae in the endocranial and dermal skeletons traversed by this duct have therefore been called the fenestra vomeronasalis interna and fenestra vomeronasalis externa respectively.

CONDITIONS IN *VARANUS*

(1) *Bones*

The skull bones of *Varanus* have been thoroughly described by Bahl (1937) with special reference to the individual bony elements and nerve foramina, and by Mertens (1942) who devoted much attention to interspecific variations. The bony skull has not, therefore, been treated in detail in the present account, although some attention has been given to certain features which have previously received little notice. No attempt has been made to make graphic reconstructions of the bones, but these elements have been drawn in from a dried skull of another juvenile specimen, so that their relationship with the chondrocranium may be appreciated (Text-figs. 1A, 2A and 3). The levels of the individual bones in these drawings have been checked as far as possible to correspond with those in the sections on which the reconstructions of the chondrocranium have been based; in some cases slight alterations in the outlines of these bones have been made. The specific features of the bony skull described have been included with the accounts of the non-bony structures related to them, while the palate has been dealt with in the comparative section.

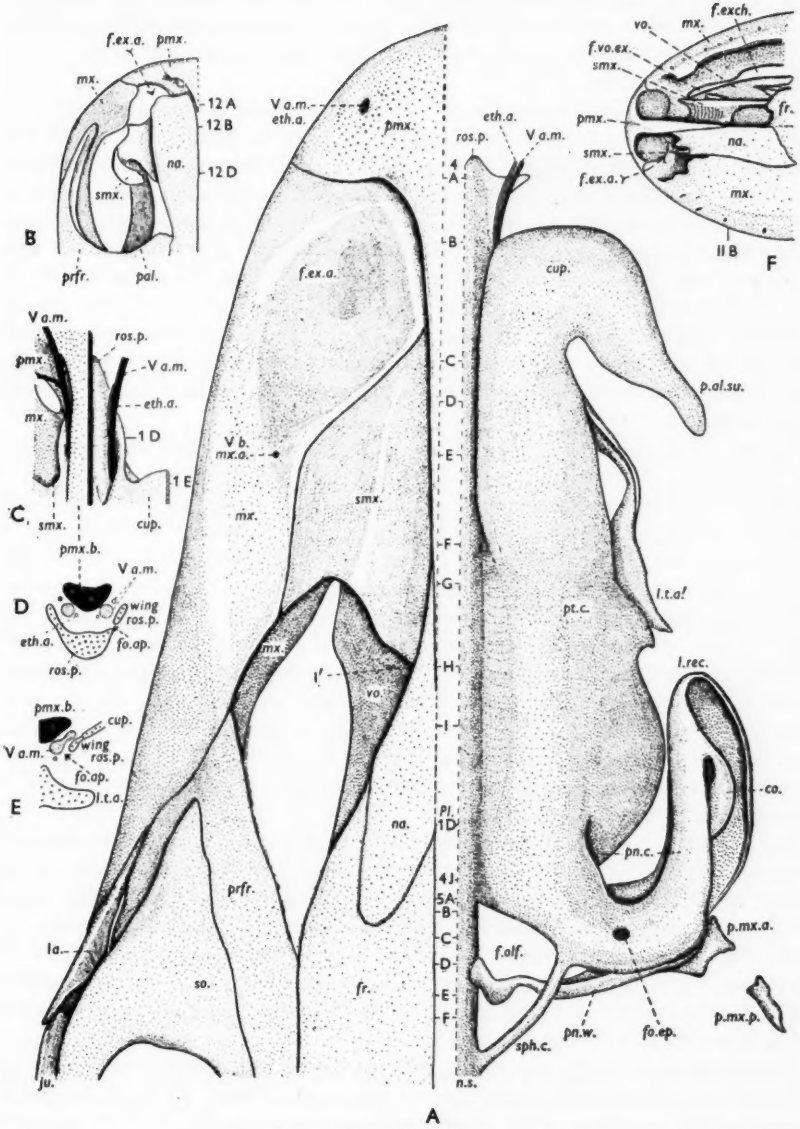
(2) *Nasal capsule*

The general morphology of the saurian chondrocranium is now well known from the work of Born (1879), Gaupp (1900), Rice (1920), de Beer (1937) and others; it is, therefore, only necessary to describe in detail the features which are particular to this genus. The reconstructions given (Text-figs. 1A, 2A and 3) agree in the main with that of Pratt. The nasal capsule is narrow and elongated, corresponding with the contours of the head. As in all living reptiles it remains unossified throughout life.

The unfenestrated nasal septum is low and terminates anteriorly in a flattened rostral process which is expanded into lateral wings. These wings are upturned, especially in *V. salvator* (Text-fig. 1D) and support the medial ethmoidal nerves and vessels. In *V. monitor* the wings taper off behind into the central part of the rostral process, which widens out again further back as the lamina transversalis anterior. The medial ethmoidal nerve and terminal branch of the ethmoidal artery run

Legend to Text-fig. 1

Text-fig. 1. A. *Varanus monitor*, subadult. Reconstruction of chondrocranium (right), and bones of snout (left), seen from above. (Bones in Text-figs. 1A, 2A and 3 drawn from dried skull.) Levels of sections in Text-figs. 4, 5 and Pl. 1D are shown. B. *Xenopeltis unicolor*, subadult. Dorsal view of bones of front of snout (drawn from dried skull). Levels of some sections in Text-fig. 12 are shown. C. *Varanus salvator*, subadult. Reconstruction of part of snout showing relationship of ethmoidal nerves and vessels to the skeleton. The main nerve branches only are shown. The right half of the premaxillary bar has been removed. In this species the medial ethmoidal nerve does not enter the premaxilla before dividing into its terminal branches, as in *V. monitor*, but runs forwards in a groove on the dorsal surface of the bone. A lateral branch passes downwards through a large foramen between the premaxilla and maxilla, while medial branches run through a foramen between the premaxillary bar and the lateral part of that bone. The foramina for the corresponding nerve branches are differently situated in *V. monitor*, and study of the figures shown by Mertens (1942) suggests that the precise relationships exhibited by the structures in this region are liable to considerable interspecific variation. D, E. *V. salvator*. Transverse sections through rostral process and premaxillary bar at levels shown in C above. F. *Tarentola mauretanica*, adult. Dorsal view of bones of front of snout, drawn from a dried skull. The nasal and part of the maxilla have been removed on one side to expose the septomaxilla and bony palate. Level of section in Text-fig. 11B is shown. (A, ×13; B, ×5; C, ×12; D, E, ×10; F, ×3·7.)



Text-fig. 1. (For explanation see p. 118.)

forwards between the medial edge of the anterior cupola and the rostral process, so that there is no distinct foramen apicale (Text-figs. 1A, 2A, 3 and 4A,B). In *V. salvator* and *V. niloticus* these relationships are slightly different. The wings join the medial edge of the anterior cupola behind, and the nerves and vessels pass through an elongated foramen apicale between the wings and central part of the rostral process (Text-fig. 1C–E).

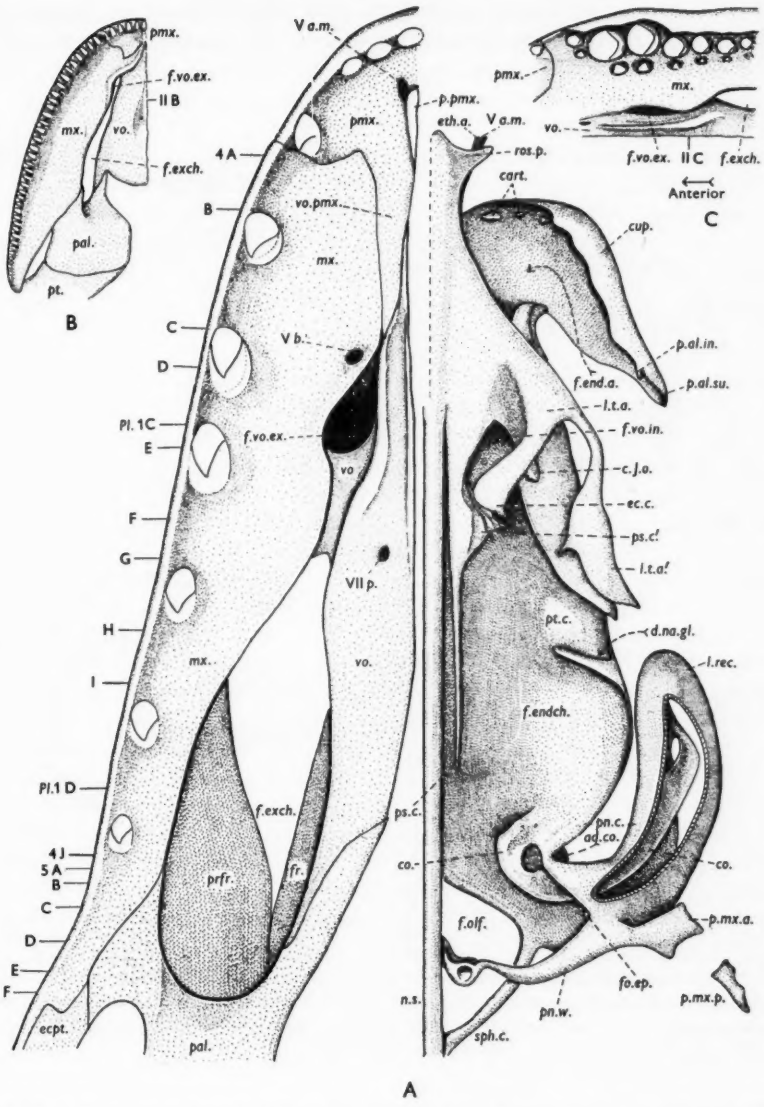
According to Pratt, the roof and sides of the nasal capsule are developed from two morphologically distinct elements—a medial element, the dorsal plate, which runs the whole length of the capsule roof on either side of the midline, and a lateral element which includes the parietotectal and paranasal cartilages which are continuous with each other. The line of junction between these medial and lateral elements cannot be determined accurately in the adult, but Pratt claims that it is indicated by the positions of the foramina for the branches of the ethmoidal nerve, and by the sites of the fenestrae superior and lateralis in those forms where they are present. In *Varanus* these fenestrae do not occur and it is only possible to infer approximately what the relative extent of these different elements may have been in the embryo. The labelling of the different regions of the capsule roof must therefore be regarded as provisional.

The front part of the capsule roof forms the large anterior cupola which lies above the curved part of the anterior nasal tube. The cupola is continuous laterally with the processus alaris superior, beneath which the anterior nasal tube passes inward to reach the nasal sac. A small projection from the cupola side wall beneath the processus alaris superior is interpreted as an inferior alar process (Text-figs. 3, 4D). The fenestra endonarina anterior curves round above this small projection, behind and beneath the processus alaris superior (Text-figs. 2A, 3).

Behind the anterior cupola the roof of the nasal capsule forms an uninterrupted covering for the elongated anterior chamber of the nasal sac. Owing to the characteristic incompleteness of the dermal skeleton of the varanid snout, this part of the capsule roof, together with the cupola, is exposed in the gap between the maxilla laterally and the narrow premaxillary bar in the midline (Text-figs. 1A, 3 and 4A–H). The nasal capsule is therefore covered dorsally only by skin and connective tissue in this region.

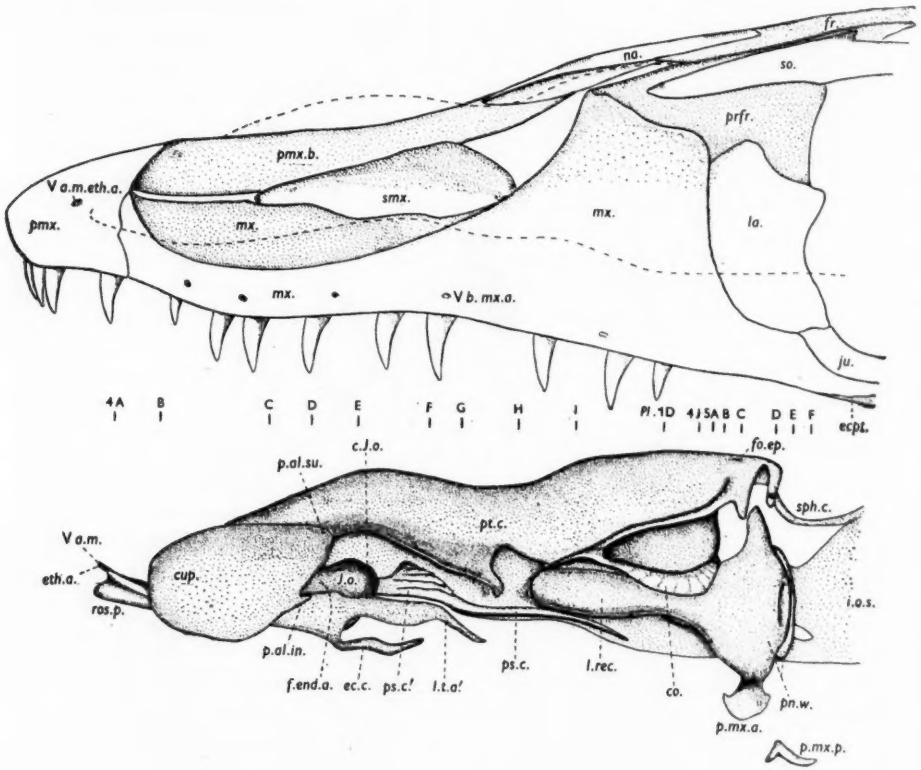
The middle part of the tectum nasi is expanded both laterally and dorsally to accommodate the olfactory chamber of the nasal sac (Text-figs. 4I–J, 5A,B). Behind this level the roof is continuous laterally with the dorsal wall of the capsule of the lateral recess. The foramen epiphaniale is situated in the transverse segment which joins the main part of the roof of the capsule with that of the lateral recess (Text-figs. 1A, 5C). The sphenethmoid commissures arise from the free posterior borders of the capsule roof (Text-figs. 1A, 5D); the olfactory lobes project over the commissures into the fenestrae olfactoria.

Behind the region of the anterior cupola, the lateral wall of the nasal capsule only extends down as far as the upper part of the nasal septum. No zona annularis is present, and the cartilage of Jacobson's organ and the whole length of the paraseptal cartilage are exposed in lateral view (Text-fig. 3). Two processes project downwards from the free edge of the lateral wall. The first of these, situated about on level with the tip of the lamina transversalis anterior, marks the termination of the lateral part



Text-fig. 2. A. *Varanus monitor*, subadult. Reconstruction of chondrocranium (right) and bones of snout (left), seen from below. Levels of sections in Text-figs. 4, 5 and Pl. 1C, D are shown. A window has been cut in the lateral recess capsule to expose the nasal concha. B. *Tarentola mauretanica*, adult. Ventral view of right side of bony palate showing paleochoanate condition. (Drawn from dried skull.) Level of section in Text-fig. 11B is shown. C. *Tupinambis nigropunctata*, adult. Ventral view of right side of bony palate showing incomplete neochoanate condition. (Drawn from dried skull.) Level corresponding to section in Text-fig. 11C is shown. (A, × 13; B, × 4; C, × 2·2.)

of the floor of the nasal capsule which runs to a point posteriorly (Text-fig. 2A). The second process lies farther back at the level of the front of the lateral recess capsule. The duct of the nasal gland (Text-figs. 4I, 10C) hooks medially round this process before entering the nasal sac.



Text-fig. 3. *Varanus monitor*, subadult. Reconstruction of chondrocranium (below) and bones of snout (above), seen from the side. Outline of roof of nasal capsule and lower edge of nasal septum is shown by interrupted lines in upper drawing. Levels of section in Text-figs. 4, 5 and Pl. 1D are shown. The prefronto-maxillary contact in the specimen drawn is unusual; in most specimens of *V. monitor* the contact runs obliquely downwards from in front, and the maxilla sends up a spur which articulates also with the frontal and tip of the supraorbital. (× 12.)

The posterior third of the lateral wall is modified in relation to the conchal infolding and the formation of the capsule of the lateral recess.* The morphology of this region was found to differ considerably in the three species of *Varanus* examined.

*This structure, which is present in many other lizards (see later) has usually been termed the cavum extra-conchale or extra-conchal recess. Since the nasal concha extends into it in *V. monitor*, however, the term lateral recess has been thought more appropriate.

In *V. salvator* the lateral recess capsule is joined with the lateral wall of the main nasal capsule, and the concha is not continued round into the recess (Text-fig. 10A). In *V. niloticus* conditions appear to be intermediate between those in *V. salvator* and *V. monitor*; the lateral recess is more extensive than in the former species and its anterior part is cut off from the rest of the nasal sac by a separate medial wall.

In *V. monitor* the lateral recess reaches its greatest development, and is completely cut off from the rest of the nasal sac for most of its length by a separate medial wall and floor (Text-figs. 4I,J, 5A,B and 10B,C). The extent of the concha inside the main part of the nasal capsule is relatively small; it continues laterally, however, and runs down most of the length of the lateral recess capsule, to the medial wall of which it is attached (Text-figs. 4I,J, 5A,B and 10B). As the roof of the recess capsule is only partly complete the anterior part of the concha is exposed from above (Text-fig. 1A).

The lateral recess capsule may be regarded as a continuation of the main nasal capsule bent round laterally, so that the medial wall of the former is continuous with the lateral wall of the latter, which in this region is derived from paranasal cartilage. It therefore seems probable that the greater part of the recess capsule is of paranasal origin, with perhaps a dorsal plate contribution to the region behind the foramen epiphaniale. The relations of the paranasal cartilage and concha to the nasal sac in *V. salvator* and *monitor* are shown diagrammatically in Text-fig. 10A,B.

The floor of the nasal capsule, in contrast with the roof, is very incomplete. Anteriorly, three small nodules of cartilage lie beneath the front edge of the anterior cupola (Text-figs. 2A, 4B). Towards the midline the rostral process sweeps backwards and laterally to become continuous with the lamina transversalis anterior. The latter structure does not join the parietotectal cartilage to form a zona annularis, but runs backwards for some distance to a free posterior tip. The cartilage of Jacobson's organ projects upwards from the lamina transversalis (Text-figs. 4D,E, 9A).

Pratt has stated that paraseptal and ectochoanal cartilages are absent in *Varanus*. Examination of further material, however, indicates that these are present in the usual saurian position. Both cartilages are more extensive in *V. monitor* than in *V. salvator* and *V. niloticus*. Near its root the paraseptal cartilage is much swollen and fragmented to form a cartilaginous network through which the vomeronasal nerve bundles pass on their way backwards from Jacobson's organ (Text-figs. 3, 9B). The ectochoanal cartilage is directed medially and extends beneath the swollen part of the paraseptal, partly enclosing the fenestra vomeronasalis interna (Text-fig. 9A). Above the ectochoanal cartilage and lamina transversalis anterior, the side wall of the nasal capsule is bent round and lies beneath the lateral part of the anterior chamber of the nasal sac (Text-figs. 4F,G) so that the floor of the capsule is duplicated in this region.

Behind the level of the ectochoanal cartilage, the floor of the nasal capsule is absent, so that the fenestra endochoanalis is very extensive. In the complete skull, however, this vacuity is partly closed by the vomer and the palatal process of the maxilla. The floor of the lateral recess capsule is continuous with the paranasal cartilage; the interpretation of this region has already been discussed. The lateral recess capsule lies against the concave inner aspects of the maxilla and prefrontal, and

is separated from the inwardly projecting maxillary shelf by the lachrymal ducts (Text-figs. 4I, J, 5A,B).

The postnasal wall (Jarvik 1942: planum antorbitale, other writers) passes from the transverse segment connecting the capsule of the main nasal sac with that of the lateral recess to the edge of the nasal septum. It is in contact with the latter, but is not fused with it. Near the septum its lower edge is continuous with a small anteriorly directed process, which, by analogy with other forms, probably represents a detached caudal rudiment of the paraseptal cartilage (Text-figs. 2A, 5E). The anterior face of the postnasal wall is slightly folded, accommodating small extensions from the antorbital space of the nasal sac. Behind the postnasal wall the prefrontal swings medially to form a rigid bony wall at the back of the nasal capsule (Text-fig. 6).

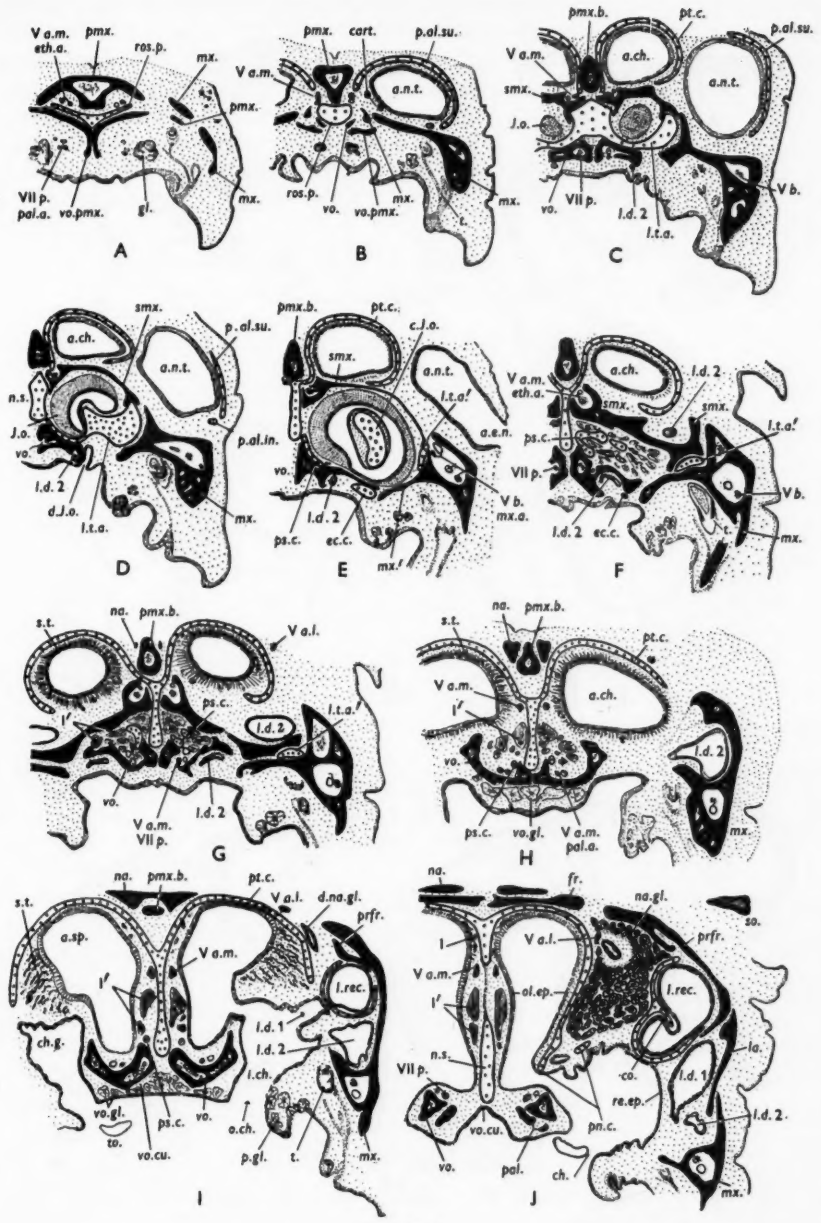
(3) *Nasal sac*

My reconstruction of the nasal sac of *V. monitor* (Text-fig. 10D) agrees closely with those shown by Wegner (1922) and Pratt (1948). The anterior external nostril is longer and situated relatively farther forward in this species than in *V. niloticus* and *V. salvator* (Text-fig. 10A,B). The anterior nasal tube is also more elongated and curved in *V. monitor*. It is possible that these differences have an adaptive significance, for *V. monitor* is a partly deserticolous form, whereas the other two species have amphibious tendencies (see Wegner, 1922: Mertens, 1942).

The anterior nasal tube passes medially to become continuous with the very elongated anterior chamber of the nasal sac. The latter is partly surrounded by spongy sinusoidal tissue (Text-fig. 4G–I). Bruner (1907) has shown that this tissue is present in many lizards, and suggests that its intumescence provides a mechanism for closing the nasal passages without interfering with the other functions of the head. The anterior chamber leads backwards into the olfactory chamber, the point of entry of the nasal gland duct indicating the region of transition between the two. The most anterior part of the olfactory chamber is known as the anterior space (Beecker, 1903), and extends ventrally, coming into relationship with the front of the choanal groove (Text-fig. 4I). The anterior space passes back into the conchal zone which is divided by the projecting concha into dorsal and ventral parts (Text-figs. 4J, 5A,B). The ventral part of the conchal zone leads downwards through the opening of the inner choana into the choanal tube, which communicates with the mouth at the outer choana (Text-fig. 10C). Behind the choanal region the nasal sac ends in a small blind pocket, the antorbital space (Text-fig. 5D,E).

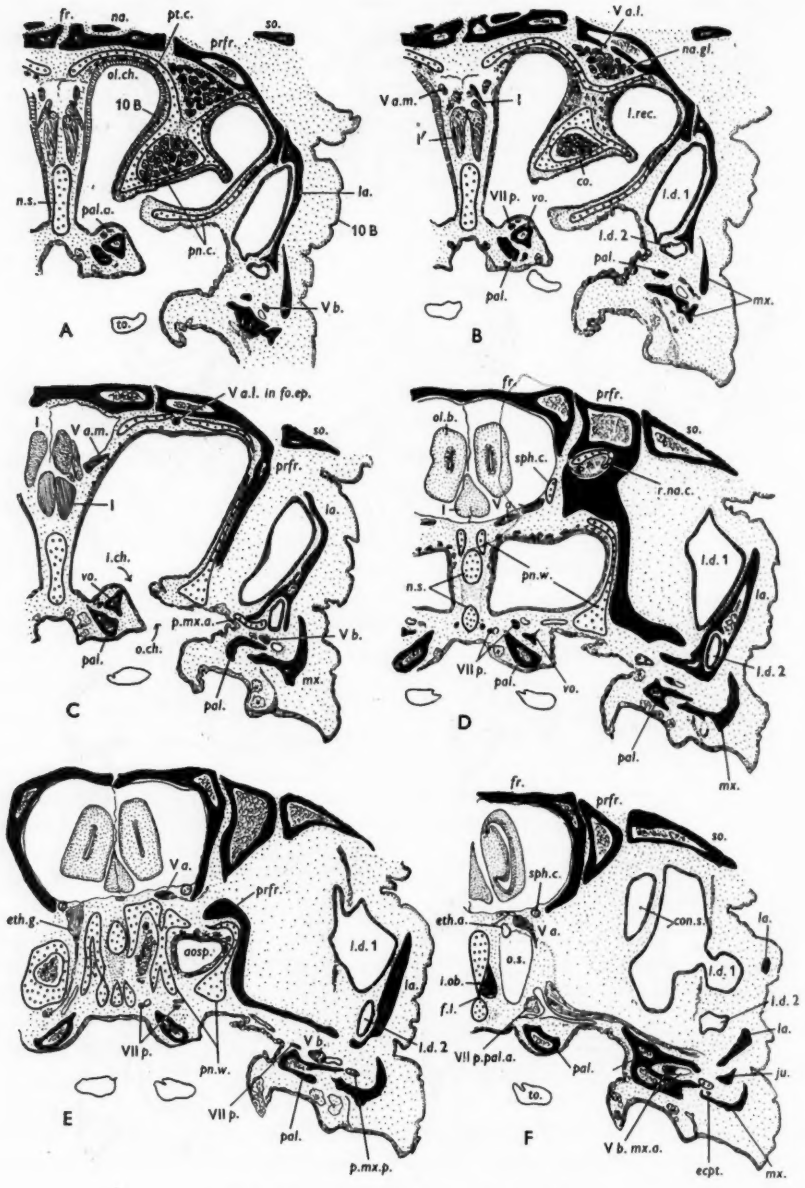
The lateral recess of the nasal sac communicates posteriorly with the main olfactory chamber (Text-fig. 5A,B) and may be regarded as a continuation of it. The variations in the extent of the lateral recess in the different species of *Varanus* studied have already been described; it seems possible that these may also be related to differences in habits.

The anterior chamber is lined by squamous stratified epithelium which continues back on to the roof of the anterior space. The floor of the latter, however, is covered by ciliated epithelium of respiratory type and this extends backwards over the walls of the ventral conchal zone to the region of the choanal tube. Here the respiratory epithelium is replaced by the stratified squamous epithelium of the palate (Text-



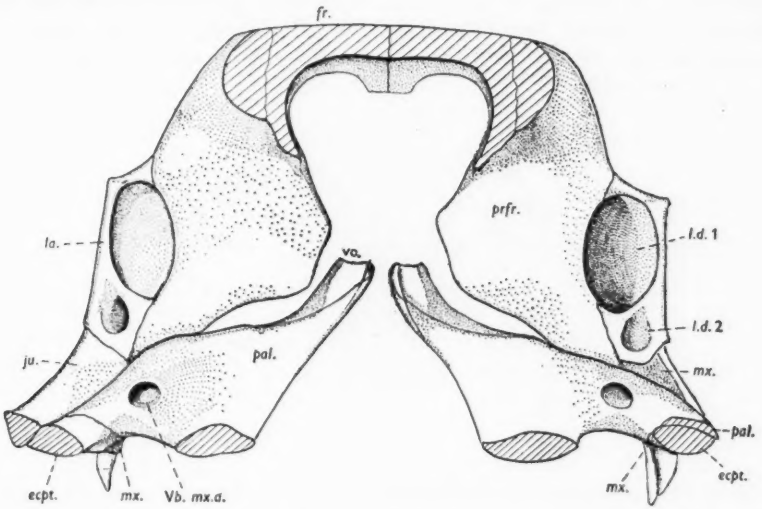
Text-fig. 4

Text-figs. 4, 5. *Varanus monitor*, subadult. Transverse sections through snout at levels shown in Text-figs. 1–3. (×11.)



Text-fig. 5. (For explanation see p. 125.)

fig. 10C). The sensory olfactory epithelium of *Varanus* is more limited in extent than in many other lizards. It is apparently confined to the dorsal surface of the conchal projection and to the roof and medial wall of the conchal zone, giving way to respiratory epithelium medially, above the inner choana (Text-fig. 10C). The olfactory fibres arise from the sensory cells of the olfactory chamber and pass medially until they reach the side of the nasal septum. They become arranged into fibre bundles which run back dorsal to the vomeronasal nerves to enter the tip of the olfactory bulb (Text-figs. 4I,J, 5A,B).



Text-fig. 6. *Varanus niloticus*, subadult. Transverse section of bony snout just in front of orbits, seen from behind. (Drawn from a dried skull.) The canal for lachrymal duct 2 penetrates the lachrymal bone; that for *l.d.* 1 passes between the lachrymal and prefrontal. Cf. Text-fig. 5E,F for relationships of non-bony structures. The jugal is missing on the right side. (× 6.)

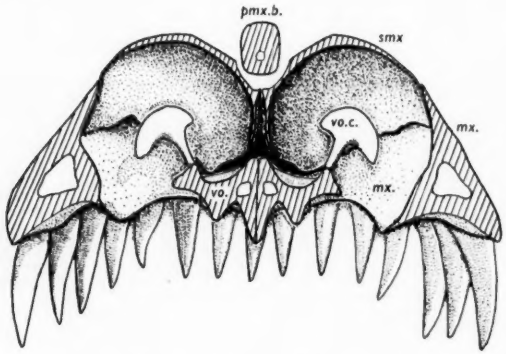
The appearance of the epithelium lining the lateral recess (Pl. 1D) varies considerably in different places. At the tip of the concha there is a small patch of respiratory type epithelium containing mucous cells. The remainder of the recess is lined by epithelium of transitional type which on the dorsal surface of the concha approaches sensory olfactory epithelium in appearance. The sensory nature of this area might be expected if the dorsal part of the recess is regarded as a continuation of the main dorsal conchal zone. No nerve fibres were, however, observed to arise from the epithelium of this or any other part of the lateral recess.

(4) *Organ of Jacobson*

The vomeronasal organs (Text-figs. 4D,E, 8; Pl. 1C) are relatively larger than in most lizards. Experimental evidence based on other squamate types (e.g. Noble & Kumpf, 1936) has shown that odorous particles are conveyed to the duct of Jacobson's organ or to the neighbouring parts of the palate by the tips of the tongue. The

presence of a long bifid retractile tongue in *Varanus*, together with the large size of Jacobson's organs, suggests that the vomeronasal sense plays as important a part in the behaviour of these lizards as has been shown to be the case in snakes.

In their general morphology the vomeronasal organs of *Varanus* conform to the usual squamate pattern as described by Pratt. Each organ possesses a large dorsal dome lined by sensory epithelium, and a mushroom body which is invaginated into the lumen from in front and below. This structure is covered by non-sensory ciliated epithelium, beneath which is a layer of rather dense connective tissue. This is supported by the cartilage of Jacobson's organ which projects upwards and backwards from the lamina transversalis anterior. The cartilage in turn rests on the vomerine concha which is carried on a stalk from the dorsal surface of the vomer (Text-figs. 7, 8 and 9).

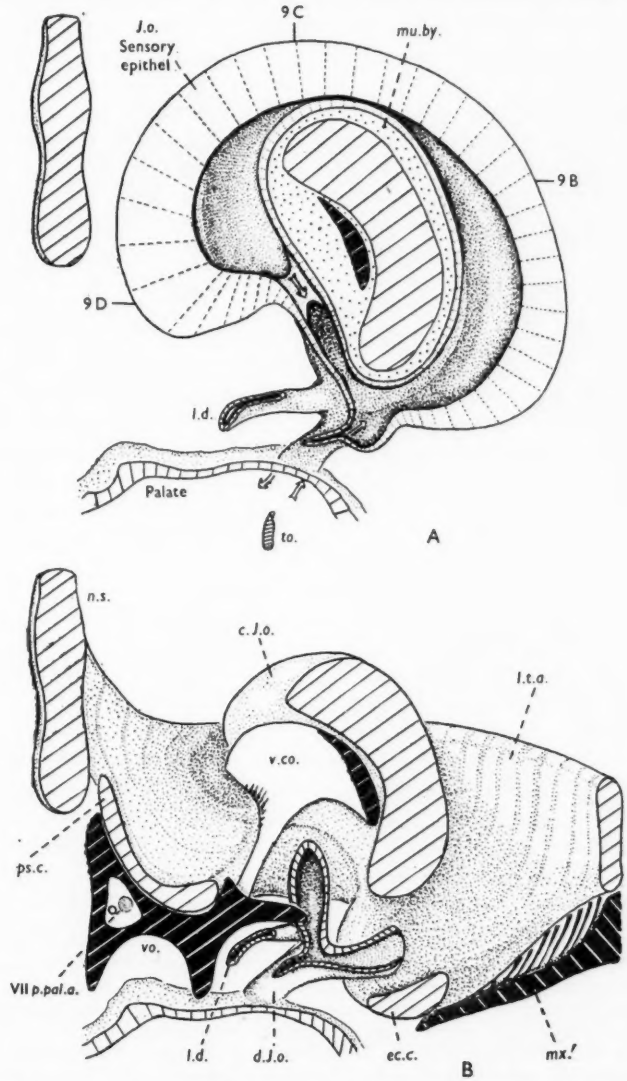


Text-fig. 7. *Varanus niloticus*, subadult. Anterior part of bony snout in transverse section seen from behind, showing chamber for Jacobson's organ. (Drawn from a dried skull.) The vomerine concha (*vo.c.*) supports the mushroom body of Jacobson's organ. Cf. Pl. 1C and Text-figs. 4E, 8 for relationships of non-bony structures. (×6.)

The spiral duct of Jacobson's organ (Text-figs. 8, 9) has a crescentic opening into the lumen beneath the posterior part of the mushroom body, and passes downwards and forwards to lead into the mouth. The lachrymal duct opens into its medial aspect as it traverses the tissues of the superficial palate. The existence of a ciliary mechanism by which odorous particles are carried into the lumen of Jacobson's organ along the lateral aspect of its duct, over the mushroom body and back into the mouth down the medial aspect of the duct has been suggested by Pratt. The direction of this one-way circuit is indicated by arrows in Text-fig. 8A. Since the duct of Jacobson's organ is lined by stratified epithelium, it would seem that odorous particles would have to be inserted into the lumen of the organ by the tongue tips before they could come under the influence of the cilia on the mushroom body.

The vomeronasal nerves are more numerous than the olfactory nerves. Their fibres arise from the sensory epithelium of the dorsal dome of Jacobson's organ and pass back over the concave upper surface of the vomer, between this bone and the septomaxilla. In this part of their course the fibres traverse the cartilaginous network formed by the swollen fragmented parts of the paraseptal cartilage (Text-

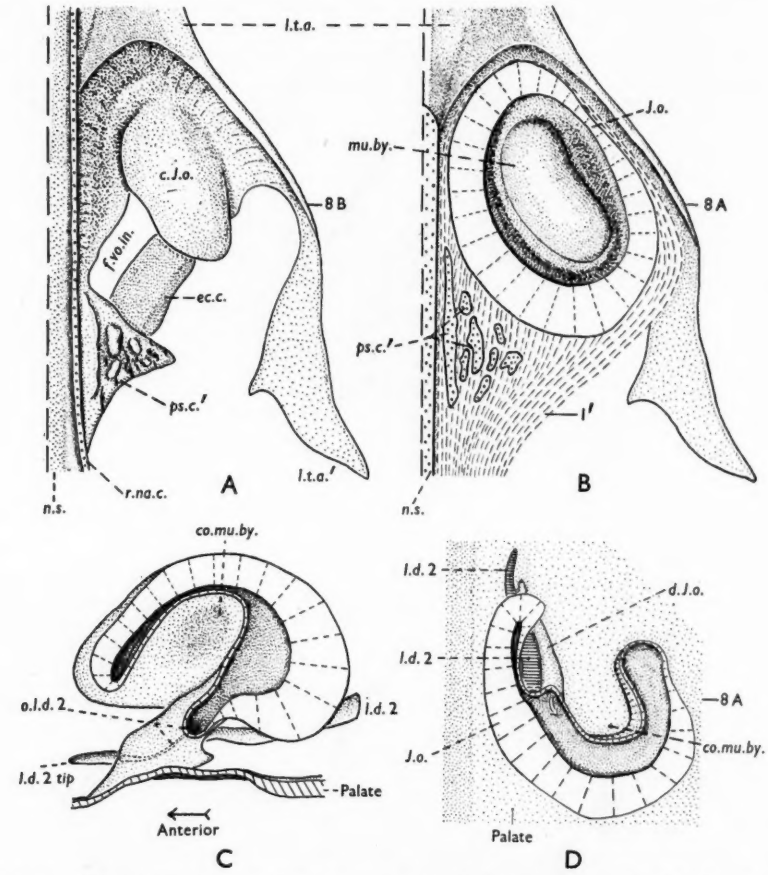
figs. 4F, 9B). The nerves then become arranged into several large bundles which run back along the side of the nasal septum to reach the accessory olfactory bulb. The vomeronasal nerves, especially near their origin from Jacobson's organ, are richly



Text-fig. 8. A. *Varanus monitor*, subadult. Reconstruction of right organ of Jacobson seen in transverse section from behind. Level of section shown in Text-fig. 9B, D. Arrows indicate probable direction of flow of particles. B. *V. monitor*. Reconstruction of cartilaginous capsule and duct of Jacobson's organ seen in transverse section from behind. Level of section as in Text-fig. 8A; level also shown in Text-fig. 9A. Some bones are included. The lachrymal duct *l.d.* figured is (l.d.) 2. (A, B, × 50.)

vascularized, each of the main fibre bundles containing a dozen or more visible arterioles in any single cross-section.

Each organ of Jacobson is almost completely surrounded by a skeletal investment from which it is separated by a layer of loose connective tissue containing many



Text-fig. 9. A. *Varanus monitor*, subadult. Reconstruction of cartilaginous capsule of Jacobson's organ seen from above after removal of roof of nasal capsule (cut edge shown). B. *V. monitor*. Reconstruction of right organ of Jacobson and cartilaginous capsule seen in horizontal section from above at level shown in Text-fig. 8A. The mushroom body is shown intact. C. *V. monitor*. Reconstruction of right organ of Jacobson seen in longitudinal section from lateral side. Level of section shown in Text-fig. 8A. Cartilage not included. D. *V. monitor*. Reconstruction of right organ of Jacobson seen in horizontal section from above at level shown in Text-fig. 8A. Cartilage not included. (All × 23.)

blood-vessels. This investment consists of an inner cartilaginous and an outer bony capsule (Text-figs. 7, 8 and 9). The cartilaginous capsule is incomplete dorsally. Its floor is formed by the hollowed posterior edge of the lamina transversalis anterior

with its projecting cartilage of Jacobson's organ, and farther back by the paraseptal and ectochoanal cartilages which extend posteriorly from the lamina. The latter cartilage projects downwards outside the bony capsule, passing between the vomer and the palatal process of the maxilla, and coming to lie in the superficial palate (Text-fig. 4E, F). The medial wall is formed by the nasal septum, while an incomplete lateral wall is provided by the free lateral extension of the lamina transversalis anterior.

The bony capsule (Text-fig. 7) is more complete, and encloses the organ of Jacobson and its cartilaginous capsule. The septomaxilla arches across the dorsal dome of the organ, separating it from the floor of the nasal sac. Laterally the septomaxilla is in contact with the vertical part of the maxilla, but medially and in front there is a gap between the septomaxilla and the dorsal surface of the vomer which is occupied by the nasal septum and the lamina transversalis anterior. These pass forwards out of the bony capsule to become continuous with the rostral process. At the posterior aspect of the bony capsule there is also a small vacuity between the septomaxilla and vomer through which pass the vomeronasal nerves.

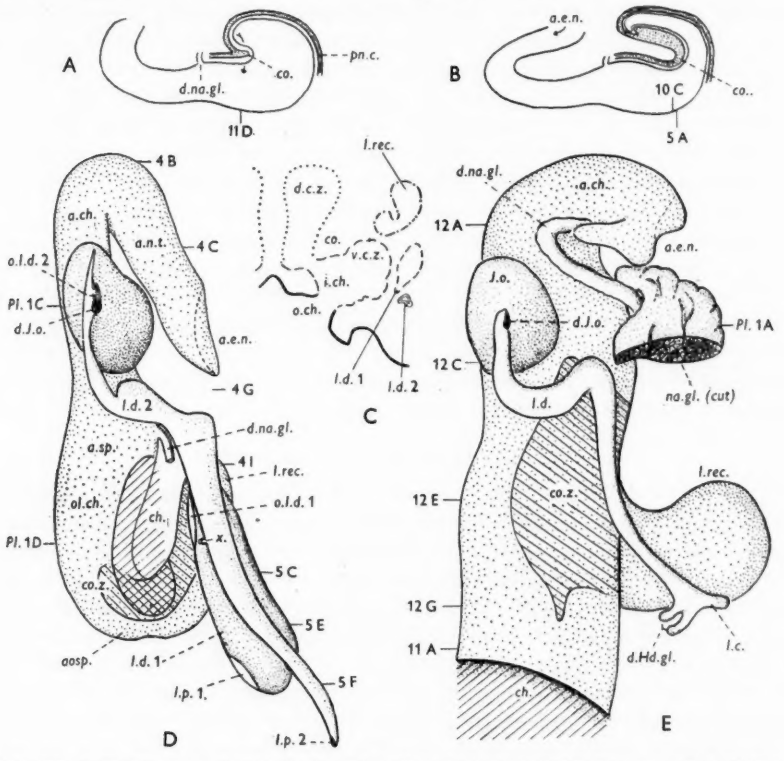
The floor of the capsule is formed by the lateral extension from the vomer and the palatal process of the maxilla: the duct of Jacobson's organ passes down between these structures (Text-fig. 8B) through the fenestra vomeronasalis externa. Posterior to this level the lateral margin of the vomer and the maxillary palatal processes come into apposition so that the fenestra vomeronasalis is completely separated from the fenestra exochoanalis. This feature will receive further attention when the palate of *Varanus* is compared with that of other types.

(5) *Lachrymal apparatus*

V. monitor (and also probably *V. salvator* and *V. niloticus*, though the condition of the sections in this region made it impossible to confirm this with certainty) differs from all other lizards examined in that there are two separate lachrymal ducts on each side. Each duct arises at a separate punctum from the medial aspect of the lower eyelid; the punctum for the upper of the two ducts (lachrymal duct 1) is placed considerably in front of that for the lower duct (*l.d.* 2) (Text-fig. 5F: Bellairs & Boyd, 1947). *l.d.* 1 is wider and much shorter than *l.d.* 2; it passes forward to enter the snout through a foramen between the prefrontal and lachrymal bones (Text-fig. 6) and passes into an elongated gutter which opens into the ventral conchal zone of the nasal sac, above the inner choana (Text-figs. 4I–J, 5A–F; Pl. 1D). From its origin to about the level where it enters its bony canal, *l.d.* 1 is lined by stratified epithelium which is continuous with that of the palpebral conjunctiva; in the anterior part of its course, however, its epithelium is of ciliated respiratory type, similar to that of the ventral conchal zone into which the duct opens.

Lachrymal duct 2 passes back beneath the punctum for *l.d.* 1 (Text-fig. 5F) and enters the snout through a foramen in the lachrymal bone (Text-fig. 7). It then runs forwards on the inner surface of the vertical part of the maxilla, resting on the bony shelf formed by the maxillary palatal process (Text-fig. 4H–J). In this situation it reaches a level a little posterior to Jacobson's organ (Text-fig. 4H), and then curves sharply inwards and downwards, passing transversely across the concave posterior border of the septomaxilla to open into the medial aspect of the duct of Jacobson's

organ. The angular part of the duct shows a marked dilatation which bulges forwards on to the dorsal surface of the septomaxilla (Text-figs. 4F, G, 10D). Anterior to this level the diameter of the duct undergoes marked reduction; its opening into the duct of Jacobson's organ is elongated in the antero-posterior direction but is compressed from above downwards, so that its orifice has the form of a long narrow slit (Pl. 1D; Text-figs. 9C, 10D). The lachrymal duct continues forwards for some distance anterior to the level at which it opens into the duct of Jacobson's organ, ending blindly about the level of the front of the organ (Text-fig. 1C).



Text-fig. 10. A, B. Diagrams showing horizontal section of nasal sac in (A) *Varanus salvator* (plane of section shown in Text-fig. 11D), and (B) *V. monitor* (plane of section shown in Text-fig. 5A). (Partly based on personal communication from C. W. M. Pratt.) Conditions in these two species illustrate the development of the lateral recess and the corresponding changes in the concha and paranasal cartilage (stippled). C. *V. monitor*. Diagram showing different types of epithelium lining nasal sac etc., at level of Text-fig. 4J. Stratified epithelium of palate shown in thick black lines; sensory epithelium over dorsal conchal zone (*d.c.z.*) in dots; ciliated epithelium with mucous cells (respiratory type) over ventral conchal zone (*v.c.z.*), upper part of choanal tube, tip of lateral recess concha and lachrymal duct 1, shown by interrupted lines; transitional epithelium with cilia but few mucous cells over most of lateral recess, shown by interrupted lines and dots; columnar non-ciliated epithelium (*l.d.* 2 only) in double lines. D, E. Diagrammatic reconstruction of nasal sac, organ of Jacobson and lachrymal ducts seen from below in (D) *V. monitor* and (E) *Xenopeltis unicolor*. Extent of choanal tube is indicated by slanting lines; conchal zone (*co.z.*) shown by slanting lines and dots. Levels of some sections in Text-figs. 4, 5, 11, and 12 and in Pl. 1A, C, D are shown.

At one point in its course, below the anterior opening of the upper duct, *l.d.* 2 communicates with the ventral conchal zone by means of a short transverse channel (Pl. 1D); this channel has a small tortuous diverticulum leading from it. In this region a small invagination of epithelium and connective tissue projects into the main lumen of the duct. The significance of these features is obscure.

In the anterior part of its course *l.d.*2 is lined by stratified epithelium. Around the level of the front of the lateral recess this is replaced by columnar non-ciliated epithelium which becomes continuous with the respiratory epithelium of the ventral conchal zone at the transverse communication (Pl. 1D). In the posterior part of its course, behind the lachrymal foramen, the epithelium again changes to the stratified type, as in the posterior part of *l.d.*1.

The curious relationship of the two lachrymal ducts to the prefrontal and lachrymal bones (Text-fig. 6) was first described by Mertens (1942), and is apparently constant for all species of *Varanus*. In all species the upper duct (*l.d.*1) is wider than the lower, but the relative disparity in size of the two ducts varies considerably.

(6) *Glands and teeth*

The nasal (lateral nasal, Fahrenholz, 1937) gland is smaller than in many lizards and is situated in the aditus conchae, between the paranasal cartilage and the medial wall of the lateral recess capsule. Its duct leads forwards, hooking medially round the posterior of the two projections from the capsule roof, to open into the nasal sac around the level at which the anterior chamber becomes continuous with the anterior space (Text-figs. 4I, J, 5A, B and 10D). Its acini are mainly mucous in character.

The distribution of the palatal glands follows the usual saurian pattern (Fahrenholz, 1937). A median vomerine group of glands lies along the lower border of the vomerine cushion, while the more scattered acini of the palatine group are situated farther laterally (Text-fig. 4I). The superior labial glands are represented only by a few isolated acini along the medial aspect of the dental lamina. The glands of the palate appear to be mainly serous in character.

The pleurodont teeth of *Varanus* vary in appearance, depending on the age and species of the individual. In many cases they are sharp and recurved: in mature specimens, particularly of *V. niloticus* and *V. exanthematus*, they may be blunt and rounded (Mertens, 1942). It is possible that the diet, which ranges from small vertebrates to insects and crabs (Cowles, 1930), may also be associated with the form of the teeth. No study of the histology or mode of tooth succession was made; a review of the relevant literature is given by Peyer (1937).

(7) *Nerves*

The topography of the cranial nerves has been described by Fischer (1852) in *Varanus bengalensis* and by Watkinson (1906) in *V. bivittatus*, while Bahl (1937) has enumerated the nerve foramina in the bony skull of *V. monitor*. A more detailed account of the cranial nerves of a lizard has been given by Willard (1915) for the iguanid *Anolis carolinensis*. My own findings largely confirm those of the workers cited, but in the absence of selectively stained material it has not been possible to investigate the distribution of nerve endings, or ganglionic connexions. The following account is based mainly on serial sections of *V. monitor*.

Apart from the nerves of special sense, the snout receives its innervation from three sources: the ophthalmic and maxillary divisions of the trigeminal, and the palatine branch of the facial nerve. The two branches of the fifth nerve probably carry fibres which are mainly somatic sensory in nature; the extensive communication between these two nerves and the palatine nerve, however, renders it likely that some autonomic fibres are also distributed with the two trigeminal branches. The palatine nerve is probably composed in the main of pre- and post-ganglionic parasympathetic fibres which are distributed to the glands and blood vessels of the snout. Sympathetic and visceral afferent fibres may also be present (Willard, 1915; Adams, 1942).

The ophthalmic nerve passes along the medial aspect of the orbit on either side of the interorbital septum, and enters the snout through the orbitonasal fissure, between the septum and the sphenethmoid commissure (Text-fig. 5F), where it becomes the ethmoidal nerve. As it lies in the fissure it possesses a swelling in which many ganglion cells can be seen. This swelling corresponds with the ethmoidal ganglion described by Willard in *Anolis*, and communicates with the palatine nerve by a band of fibres which runs vertically downwards behind the postnasal wall (Text-fig. 5E).

Slightly anterior to the ethmoidal ganglion the nerve divides into its lateral and medial rami (Text-fig. 5D). The lateral ramus passes forwards beneath the roof of the nasal capsule for a short distance, and then leaves the capsule through the foramen epiphaniale (Text-fig. 5C). It then breaks up into numerous branches among the acini of the nasal gland (Text-fig. 5B, etc.) and probably supplies the latter (see Gaupp, 1888) as well as the skin and tissues of the snout.

The medial branch of the ethmoidal nerve passes forwards at the side of the nasal septum where it is closely related to the olfactory and vomeronasal nerves (Text-figs. 4, 5A–C). It enters a groove along the medial edge of the septomaxilla and runs forwards in this situation (Text-fig. 4D–G), escaping through a foramen at the front of the bone (Text-fig. 4B,C). Anterior to this level it lies between the rostral process and roof of the nasal capsule (Text-fig. 4B), and in front of this again, between the rostral process and premaxilla; the slightly different condition in *V. salvator* has been described. The nerve then enters the posterior premaxillary foramen (of Bahl) and divides into dorsal and ventral branches which emerge from the dorsal and ventral premaxillary foramina respectively (Text-figs. 1A, 2A) to supply the tip of the snout. No branches supplying the nasal sac or the organ of Jacobson could be identified, although the existence of such branches seems probable.

The maxillary nerve passes along the ventrolateral aspect of the orbit and enters the snout through a canal in the palatine bone (Text-figs. 5F, 6). During its course through the orbital region it has two principal communications with the palatine nerve: one near the back of the orbit and another just anterior to the orbit. After the nerve has left the palatine canal (Text-fig. 5E) it enters the maxillary canal after giving off several small branches which are probably distributed to the glands and epithelium of the palate. At intervals throughout its course in the maxillary canal the nerve supplies the teeth, and also sends branches laterally which pass through small foramina in the bone and are distributed to the side of the jaw. A few rami also pass medially through the palatal process of the maxilla to reach the palate. One rather large branch emerges from the easily recognizable anterior maxillary foramen (Bahl) which lies lateral to the fenestra vomeronasalis externa (Text-

fig. 2A). Another large branch also passes upwards and medially through a foramen in the dorsal aspect of the maxilla (Text-figs. 1A, 4E), and is apparently distributed to the anterior nasal tube. The main nerve trunk finally escapes through a foramen near the anterior extremity of the maxilla and breaks up into branches in the superficial tissues of the snout.

The palatine branch of the facial nerve leaves the parabasal canal and passes forwards beneath the orbit at first medial to the pterygoid and then dorsal and lateral to it. In this situation, a little behind the midorbital region, it communicates with the infraorbital (maxillary) nerve, and bears a swelling containing ganglion cells. This swelling corresponds in position with the palatine ganglion in *Anolis*, and with the sphenopalatine ganglion figured by Adams (1942) in *Lacerta viridis*. Anterior to this level the nerve continues above the palatine bone, sending branches to the superficial palate. At the front of the orbit the nerve swings medially and enters the snout near the lower edge of the interorbitonasal septum (Text-fig. 5F). In this situation the nerve carries another ganglionic swelling which is connected with both the ethmoidal ganglion above and the maxillary nerve laterally; a small bundle of fibres joins the maxillary nerve and passes with it into the maxillary canal (Text-fig. 5E). This anterior ganglion probably corresponds with the structure described by Haller v. Hallerstein (1937) in *Anguis fragilis*. After giving off several lateral branches to the superficial palate the main trunk of the nerve passes forwards above the vomeropalatine articulation (Text-fig. 5A–D) and then over the concave upper surface of the vomer (Text-fig. 4G–I). In the region of Jacobson's organ it becomes completely enclosed in a canal in the medial part of the vomer, from which it sends at least one branch downwards through the bone to reach the superficial palate (Text-fig. 4G), probably supplying the palatine glands and epithelium (Gaupp, 1888). Anteriorly the nerve leaves the vomerine canal and breaks up into fine branches in the premaxillary region of the superficial palate.

No fibres from the trigeminal (or facial) nerve supplying Jacobson's organ could be identified, nor have any been described by Watkinson, nor by Willard in *Anolis*. It seems likely, however, that Jacobson's organ receives some sensory trigeminal innervation; it is unfortunate that in *Anolis*, the only form of which selectively stained preparations have been studied, the organ of Jacobson is smaller and less conspicuous than in most lizards. The nervus terminalis could not be identified in *Varanus*.

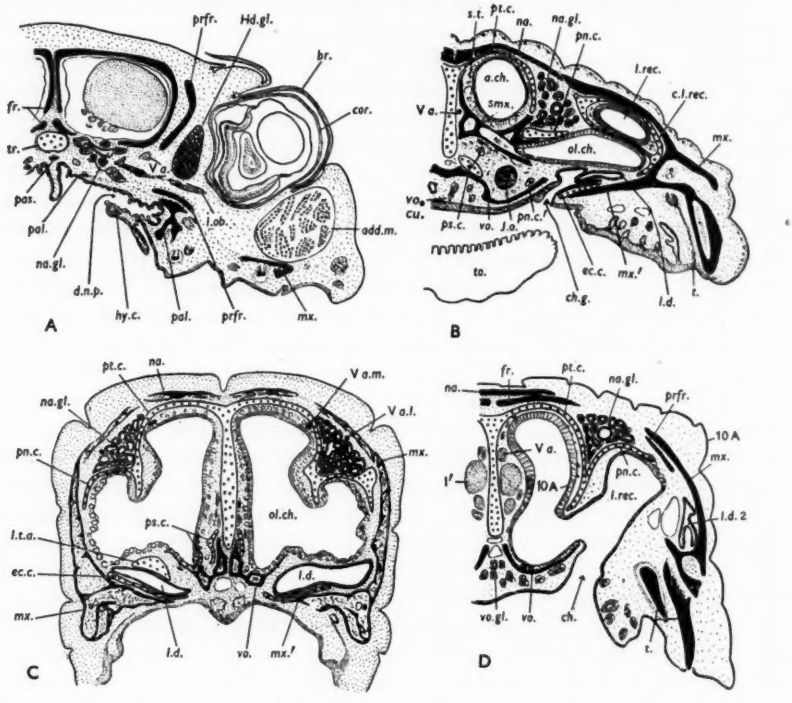
(8) *Blood vessels*

The vascular system of *V.* (*'Psammosaurus'*) *griseus* was studied by Corti (1847), but he hardly deals with the vessels of the snout. Subsequent descriptions of the blood supply of the snout in lizards have been given by Bruner (1907), who dealt only with the veins, and by Hafferl (1933) in the Bolk Handbook, while O'Donoghue (1920) has described the condition in *Sphenodon*.

The arterial supply of the snout is derived from three main vessels which all stem ultimately from the internal carotid: the supraorbital, infraorbital and palatine arteries.

The supraorbital artery in *Varanus* seems to be smaller than in *Sphenodon*. It arises from the temporal artery behind the orbit and passes forwards above the eye, lying at first lateral to the taenia marginalis and then running downwards and medially on the lateral aspect of the downgrowth from the frontal bone. It enters

the snout through the orbitonasal fissure in proximity to the ethmoidal nerve, and becomes the ethmoidal artery (Text-fig. 5F). The artery then breaks up into a large number of dorsal and ventral branches behind the postnasal wall which run forwards to supply the nasal sac and the spongy tissue around the anterior chamber. One large ventral branch runs downwards and laterally to anastomose with the palatine



Text-fig. 11. A. *Xenopeltis unicolor*, subadult. Transverse section through orbital region at level indicated in Text-figs. 10, 13, showing ductus nasopharyngeus. B. *Tarentola mauretanica*, adult. Transverse section of snout through posterior part of Jacobson's organ at level indicated in Text-figs. 1F, 2B, showing paleochoanate type of palate. C. *Tupinambis teguixin*, subadult. Transverse section through snout behind Jacobson's organ at level indicated in Text-fig. 2C, showing incomplete neochoanate type of palate. D. *Varanus salvator*, subadult. Transverse section through snout at level corresponding with that shown in Text-fig. 4J of *V. monitor*. The lateral recess is not closed off in *V. salvator*, and *l.d.*1 is not seen at this level. Plane of Text-fig. 10A shown. (A, × 13·5; B, × 18; C, × 7; D, × 9.)

and maxillary arteries (Text-fig. 5E, F). Some of the dorsal branches appear to supply the tips of the olfactory bulbs as well as the olfactory nerves. A large branch on each side passes forwards to enter the septomaxillary canal with the medial ethmoidal nerve, and is distributed with it (Text-fig. 4F).

The infraorbital artery also arises from the temporal artery and runs forwards on the ventrolateral aspect of the orbit, near the infraorbital (maxillary) nerve. At the front of the orbit it receives anastomotic connexions from the palatine and ethmoidal arteries and enters the maxillary canal with the maxillary nerve, being distributed

with it to the teeth, jaws, palate and sides of the snout and, through its dorsal branch, to the spongy tissue around the anterior chamber of the nasal sac.

The palatine artery arises directly from the internal carotid and passes along the roof of the mouth near the palatine nerve. In the snout it sends numerous branches to supply the palatine glands and epithelium. A large branch on each side runs forwards to enter the vomerine canal with the palatine nerve. This sends many large branches to supply the organ of Jacobson and vomeronasal fibre bundles (Text-fig. 4G, etc.).

The complicated system of venous sinuses in the saurian head has been thoroughly described by Bruner (1907) in *Lacerta agilis* and other forms: it was not possible to make any critical investigation of these structures in my own material. Bruner's observations show that almost all the venous drainage of the snout passes through an extensive series of nasal and palatine veins into the large orbital sinus (Text-fig. 5F), and ultimately into the internal jugular veins.

COMPARISON WITH OTHER LIZARDS

A detailed survey of the saurian snout and the extensive literature concerned with it is beyond the scope of this account; it is only possible, therefore, to touch on those features which seem relevant to the affinities of *Varanus*, or appear of general morphological interest.

The curiously incomplete condition of the dermal skeleton of the varanid snout does not seem to occur in other living lizards. In most forms the fenestra exonarina anterior is relatively small, and is bounded medially and in front by the premaxilla, and laterally by the maxilla which conceals the septomaxilla almost entirely. Such a condition is present in the gecko *Tarentola mauretanica* (Text-fig. 1F). In *Varanus* the maxilla does not conceal the septomaxilla in this way and the greater part of the latter bone is exposed in the huge fenestra exonarina when the nasal capsule has been removed (Text-fig. 1A). The only forms which seem to approach the varanids in this respect are the Cretaceous mosasaurs. This region has been clearly described and figured by Camp (1940) in *Kolposaurus*, where the long slender premaxillary bar and the exposed septomaxillae are highly reminiscent of the varanid condition. The septomaxilla is, however, narrower and more elongated in the mosasaur, and it may be supposed that the bony chamber for Jacobson's organ was less spacious in the sea lizard than in *Varanus*.

The palate of *Varanus* is remarkable for the completeness of both its bony and superficial components. In an extensive study of the bony palate of Sauria, Lakjer (1927) has shown that three main varieties may be distinguished. In the first type the palate is only complete as far back as the anterior margin of the fenestra vomeronasalis externa which is confluent with the fenestra exochoanalis. This type (called by Lakjer paleochoanate) is present in forms such as agamids, iguanids and geckos (e.g. *Tarentola*, Text-figs. 2B, 11B). It is regarded as the most primitive variety of saurian palate, and approximates to the condition in *Sphenodon* and the early eosuchian reptiles. An advance on this type of palate is shown by the incomplete neochoanate condition which occurs in forms such as skinks, lacertids and teiids (e.g. *Tupinambis*, Text-figs. 2C, 11C). Here the fenestra vomeronasalis externa is partly cut off from the fenestra exochoanalis by the palatal process of the

maxilla, which extends medially beneath the lateral margin of the vomer (Text-fig. 11C). The final complete neochoanate condition is found only in the genus *Varanus*, where the palatal process of the maxilla is still more extensive, and actually contacts the vomer for a short distance behind the fenestra vomeronasalis, separating it from the fenestra exochoanalis (Text-figs. 2A, 4E–G).*

These variations correspond more or less closely with the condition of the superficial palate, which forms the subject of a most extensive German literature (see Barge, 1937, for review). In the paleochoanate and incomplete neochoanate forms the choanal tube is continuous anteriorly with a long choanal groove which passes upwards towards the nasal sac, separating the median vomerine part of the palate (vomerine cushion: see Text-fig. 11B) from the lateral maxillary portion. In many forms the lachrymal duct discharges into the choanal groove, but in others, such as *Varanus* (Pl. 1C) and *Tupinambis* (Pl. 1B) it opens directly into the duct of Jacobson's organ. These relationships are to a large extent dependent upon the degree of fusion between the frontonasal and maxillary processes during ontogeny. It is hoped that some account of the embryological processes involved, together with the numerous variations in this region which occur throughout the Sauria, will be given in greater detail elsewhere.

Pratt (1948) has suggested that the presence of these choanal grooves provides a mechanism for the filling of Jacobson's organ, and that particles deposited in them by the tongue tips are carried forwards by ciliary action into the lumen of the organ. In *Varanus* these choanal grooves are relatively short, and do not extend forwards to reach the duct of Jacobson's organ. It is, therefore, assumed that the particles are inserted inside the orifice of the duct, or actually into the lumen of the organ. This mechanism, which probably also occurs in snakes where the anatomical relationships are very similar, is reputed to be more effective than the method which is dependent on the action of cilia in the choanal grooves. It may be noted that the long retractile tongue of *Varanus*, which has been regarded as suggesting affinities with snakes, is also present in the large American teiid *Tupinambis*. Although the bony palate of this form is incompletely neochoanate, the morphology of the superficial palate, with short choanal grooves and the lachrymal duct opening directly into the duct of the organ of Jacobson (Pl. 1B) approaches the varanid condition. A rather similar state is also present in some of the Amphisbaenidae, a group of specialized burrowing reptiles usually classified with the Sauria.

The general appearance of the nasal capsule, with its elongated form, incomplete floor and (in *Varanus monitor*) enormous lateral recess bears a certain resemblance to the condition in *Eumeces quinquelineatus* as described by Rice (1920), and perhaps also to that in certain agamids (e.g. *Agama atricollis*, Pratt, 1948). It would be rash, however, to suggest that these features have any great systematic value in view of the striking interspecific differences within the genus *Varanus*.

The long rostral process with its lateral wings extending forward of the nasal cupola, and the low anterior part of the nasal septum are unusual. The absence of

* It may be noted that the small 'secondary palate' formed in this way differs from the condition in crocodiles and in mammals. In these forms the secondary palate is derived entirely from the lateral bones (maxillae, palatines, etc.) which fuse in the midline, displacing the vomers upwards in relation to the nasal septum.

a discrete foramen apicale in *V. monitor* and the relationships of this foramen in *V. salvator* are also noteworthy.

Another feature of interest is the disposition of the paraseptal cartilage and the way in which the ectochoanal cartilage runs backwards and medially to extend beneath it. In many lizards (e.g. *Lacerta agilis*, Gaupp, 1900; *Eumeces quinquelineatus*, Rice, 1920) the ectochoanal cartilage projects backwards and outwards. Its inward direction in *Varanus* almost encloses the fenestra vomeronasalis interna, separating it from the fenestra endochoanalis in the same way as the vomeromaxillary contact separates the fenestra vomeronasalis externa from the fenestra exochoanalis.

The significance of the fragmentation of the paraseptal cartilage into a network surrounding the vomeronasal nerves is obscure. This feature was present in all the three species of *Varanus* studied, and also occurs in the genus *Gerrhosaurus* (Malan, 1941), in *Tupinambis teguixin*, and, to a lesser extent, in *Lacerta vivipara*.

COMPARISON WITH SNAKES

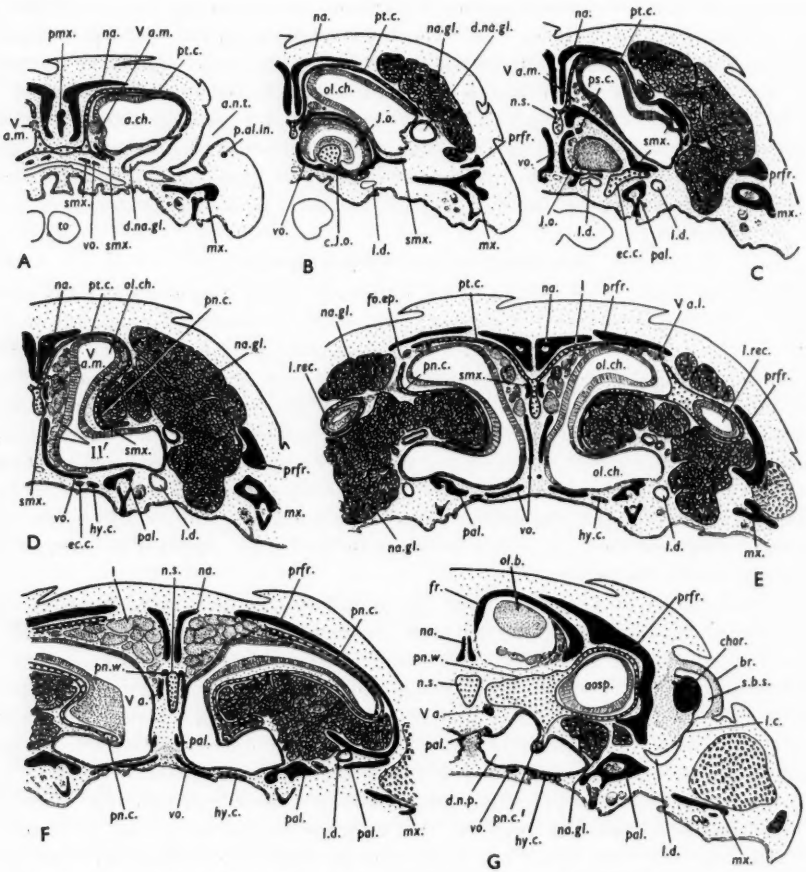
The Sunbeam snake, *Xenopeltis unicolor* Reinwardt (fam. Xenopeltidae), has been selected for comparison with *Varanus*. Two series of sections through the nasal region of this primitive and comparatively generalized ophidian have been available for study; for the specimens from which these have been prepared I am indebted to Dr Malcolm Smith, and to Mr M. W. F. Tweedie of the Raffles Museum, Singapore.

The snout of *Xenopeltis* is shorter and wider than is the case with most lizards, and contrasts particularly with the elongated snout of *Varanus*. The dorsal surface of the skull (Text-fig. 1B) shows a large fenestra exonarina anterior in which the septomaxilla is widely exposed. The relationships of the bones are so different from those in *Varanus*, however, that the latter feature can hardly be regarded as suggesting affinity between the two forms.

The bony palate of *Xenopeltis* (Text-fig. 13) differs even more strikingly from the saurian condition. The premaxillae form a rounded arcade which is in contact with the anterior tip of the maxilla on each side. This condition is found only among the most primitive snakes, and in more advanced forms such as the colubrids and vipers the premaxillae are much reduced and are not in contact with the maxillae. In all non-viperine snakes* the lateral extent of the maxilla is reduced and the bone has the form of a narrow rod which is separated from the vomer and palatine medially by a considerable gap in which the septomaxilla is widely exposed. The fenestra vomeronasalis externa lies between the septomaxilla laterally and the vomer medially, instead of between the maxilla and the vomer, as in *Varanus* and other lizards. A peculiar feature in *Xenopeltis* is the presence of a long slender process which runs forward from the main part of the palatine bone and lies above the vomer in the medial wall of the ductus nasopharyngeus (Text-figs. 11A, 12F,G and 13). The position of the fenestra exochoanalis in *Xenopeltis*, as in snakes generally, is very different from that in Sauria. In the latter the fenestra occupies a large part of the bony palate and is situated between the maxilla and the vomer, with the palatine behind (Text-fig. 2A,B,C). In snakes the fenestra is directed backwards from above the posterior part of the vomer (Text-fig. 11A) and is hardly visible from the ventral

* In the vipers the maxilla is short and highly specialized in association with the mechanism of fang erection.

aspect. The fenestra vomeronasalis externa is therefore separated by the greater part of the length of the vomer from the fenestra exochoanalis. An extensive bony 'secondary palate' is therefore present, but this lies only beneath the medial part of the nasal sac which extends laterally across the gap between the vomer and the palatine.

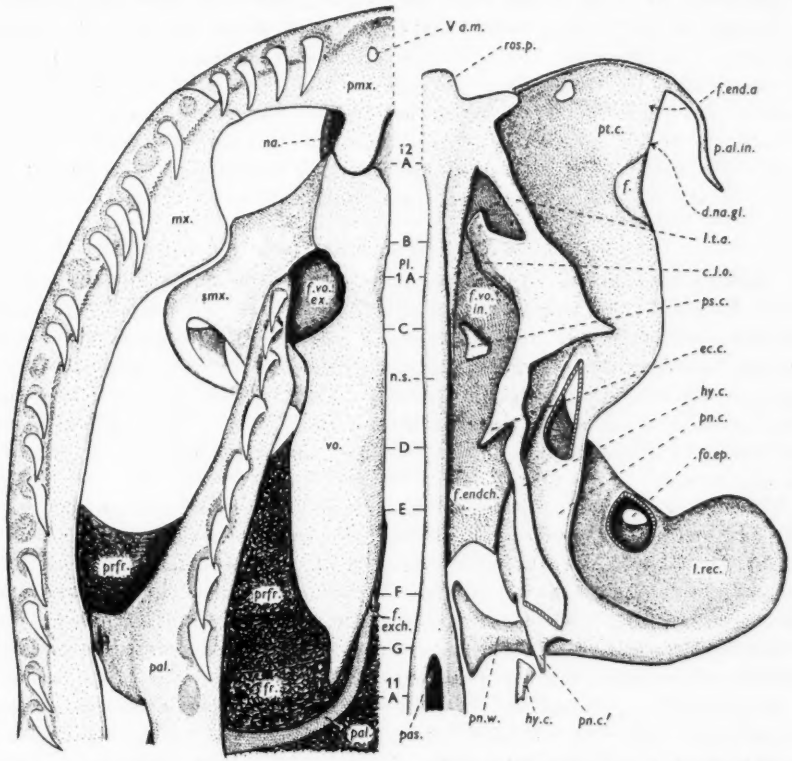


Text-fig. 12. *Xenopeltis unicolor*, subadult. Transverse sections through snout at levels shown in Text-figs. 10E and 13. (×15.)

The appearance of the nasal sac in *Xenopeltis* with its short anterior nasal tube and poorly differentiated lateral recess is shown in Text-fig. 10E. As in all snakes the choanal tube is drawn out into an elongated ductus nasopharyngeus which lies dorsal to the posterior part of the vomer (Text-fig. 11A). A ductus nasopharyngeus is also present in many skinks and certain other lizards, but here the bony relationships are very different (see Lakjer, 1927; Barge, 1937).

The most striking similarity between *Varanus* and the snakes is shown by the

condition of Jacobson's organ, by the relationship of its duct to the lachrymal system, by the morphology of the superficial palate, and by the presence of a slender bifid tongue. These structures, together with the eye glands which provide the secretion in which the odorous particles deposited by the tongue tips are carried through Jacobson's organ, constitute a functional 'vomeronasal unit' which seems to have reached its greatest development among Squamata in the varanid lizards and the snakes. In the latter a single lachrymal duct only is present. It will be seen, however



Text-fig. 13. *Xenopeltis unicolor*, subadult. Reconstruction of chondrocranium (right) and bones of snout (left) seen from below. (Bones drawn from dried skull.) Levels of section in Text-figs. 11 and 12 are shown. Windows have been cut in the paranasal cartilage and lateral recess capsule and a segment of the hypochoanal cartilage has been removed. (×25.)

(Text-fig. 10E), that the relationships of the anterior part of this structure, with its dilated angular portion behind the organ of Jacobson, and its direct opening into the duct of the latter organ, are essentially similar to those of the long lachrymal duct 2 of *Varanus*. The posterior (orbital) end of the lachrymal duct system is markedly different in *Varanus* and *Xenopeltis*, however, conditions in the former following the usual saurian pattern with the lachrymal puncta arising from the conjunctival space on the deep aspect of the lower eyelid (see Bellairs & Boyd, 1947).

The nasal capsule of *Xenopeltis* differs little from that of more advanced and 'typical' snakes such as *Natrix* (*Tropidonotus*) *natrix* (Bäckström, 1931). Characteristic ophidian features are the high anterior cupolae joined by a flattened rostral process which runs back into the low nasal septum, the long processus alaris inferior and the extensive conchal infolding which is exposed from below by the almost complete absence of the true capsule floor. The ectochoanal cartilage is connected with a long hypochoanal cartilage which runs back to support the ductus nasopharyngeus. As in snakes generally, the sphenethmoid commissures are absent.

Perhaps the only feature in the nasal capsule of *Xenopeltis* which can be regarded as primitive is the presence of a small nodule of cartilage on each side of the nasal septum behind the fenestra vomeronasalis interna (Text-figs. 12C, 13). From its position and relationship to the vomeronasal nerves, this nodule appears to represent a rudiment of the paraseptal cartilage of lizards. No similar structure has been reported in other snakes, nor have I found anything resembling it in sections of other forms usually regarded as primitive (e.g. *Cylindrophis*, *Eryx*, *Constrictor*).

DISCUSSION

In assessing the affinities of any form it is always a matter of difficulty to distinguish between those resemblances with other types which are indicative of true phylogenetic relationship, and those which result from parallel evolution. This is particularly the case with a group like the Squamata, where extensive parallel evolution has occurred in association with a host of divergent specialized trends. It is, therefore, unwise to attempt even a provisional assessment of the relationships of *Varanus* without some consideration of a wider range of characters than has been described in this account.

The striking differences between the snout of *Varanus* and that of other lizards can be matched with certain peculiarities shown by other regions of the body. The presence of cartilage canals in the epiphyses of the long bones, a feature unique among reptiles (Haines, 1941), together with other characters listed by Camp (1923) are suggestive of a long evolutionary history distinct from other living saurian families. Relationship with the mosasaurs, however, is indicated by numerous features of cranial morphology (see Camp, 1940).

In spite of its peculiarities, the basic morphology of the varanid snout, as exemplified by such structures as the bony palate and nasal capsule, are essentially saurian rather than ophidian in nature. Apart from the slightly ophidian appearance of the anterior part of the nasal septum and its relationship with the nasal cupolae and site of exit of the ethmoidal nerves, the few absolutely characteristic features of the ophidian nasal capsule are not shown, or even approached, in *Varanus*. Such typical ophidian features are the long processus alaris inferior, the presence of the hypochoanal cartilage, and the absence (or marked reduction) of the paraseptal cartilage.

The undoubted resemblances between the varanid superficial palate and vomeronasal apparatus do not seem of great phylogenetic significance. The fact that these resemblances involve the soft parts only, and are superimposed upon skeletal structures of a very different type, is suggestive of parallel evolution associated with the development of a highly efficient vomeronasal sense. The similarity between the

vomeronasal organs and palate of *Varanus* and those of at least two very distinct squamate genera, *Amphisbaena* and *Tupinambis*, lends further support to this view.

An investigation of the endocranial brain-case in reptiles shows that one of the most clearly marked distinctions between the cranial anatomy of lizards and snakes lies in the disposition of the trabeculae in the orbital region, and in the presence or absence of sphenolateral cartilages. The varanid brain-case with its high interorbital septum and fenestrated sphenolateral is essentially similar to that of such generalized lizards as *Eumeces* and *Lacerta*, and is in striking contrast with the platytrabic condition of snakes where the sphenolateral cartilages are absent in the adult state. Other features of the varanid skull such as the presence of the lachrymal and jugal bones and the structure of the ear seem to show that, on grounds of cranial morphology at least, there is no reason to regard *Varanus* as having any specially close affinities with the snakes. Indeed, certain other saurian groups appear to show a closer resemblance to snakes in many important respects (see Bellairs, 1949).

On the classification devised by Camp (1923) the Varanidae appear as the closest living relatives of snakes, and this concept has been expressed more emphatically by Romer (1945), who writes that the snakes are 'obviously derived from varanid types'. Camp's original view seems to have been mainly based on the structure of the hemipenes and vertebrae, together with certain cranial features, which, as I hope to show elsewhere, are open to a different interpretation. I do not feel that this view has been adequately substantiated by the study of cranial morphology, which has so far failed to reveal any significant resemblances between *Varanus* and the snakes which cannot as readily be explained on grounds of parallel evolution. It is therefore suggested that further evidence is required before a specially close relationship between the Varanidae and the snakes can be established.

SUMMARY

1. The anatomy of the snout of *Varanus* is described, largely from serial sections of the species *monitor*, *salvator* and *niloticus*. The nasal capsule, nasal and vomeronasal organs, lachrymal apparatus and palate are described in some detail, and an account is also given of some features of the bony snout, and the distribution of the main nerves and arteries. The terminology devised by Jarvik (1942) for the different parts of the ethmoidal region of anamniotes has been applied and extended. The most important features shown by *Varanus* are listed below.

2. The nasal capsule is incompletely covered by dermal bones on the dorsal surface of the snout. The fenestra exonarina anterior is very large, with the septomaxilla widely exposed in it. (Condition approached by mosasaurs.)

3. The nasal capsule is elongated with an unfenestrated roof. The nasal septum ends anteriorly in a flattened rostral process which extends forwards in front of the cupolae. The floor of the nasal capsule is largely incomplete and the zona annularis is absent. Paraseptal and ectochoanal cartilages are present and almost enclose the fenestra vomeronasalis interna. There is an extensive separate capsule for the lateral recess of the nasal sac in *V. monitor*. (This feature is present in some other lizards such as *Eumeces*, but is less well developed in *Varanus salvator* and *V. niloticus*.)

4. The anterior nasal tube is long and curved and the anterior chamber of the nasal sac is elongated. The olfactory chamber is limited in extent. The nasal concha

extends into the lateral recess in *V. monitor* only. The choanal opening is limited to the posterior part of the nasal sac, but is not drawn out to form a ductus naso-pharyngeus as in some lizards and all snakes.

5. The organ of Jacobson is very large and its duct opens into the mouth and receives the anterior orifice of lachrymal duct 2.

6. There are two separate lachrymal ducts on each side. Lachrymal duct 1 (arising from the superior punctum) is short and discharges into the olfactory chamber of the nasal sac after passing between the prefrontal and lachrymal bones. Lachrymal duct 2 is long and passes through a foramen in the lachrymal bone to discharge into the medial aspect of the duct of the organ of Jacobson. The relations of lachrymal duct 2 somewhat resemble those of the single lachrymal duct of snakes.

7. The bony palate is completely neochoanate, with the fenestra vomeronasalis externa separated from the fenestra exochoanalis by the vomeromaxillary contact. The superficial palate is remarkably complete, with short choanal grooves.

8. A brief description of some of the features of the snout of the primitive snake *Xenopeltis unicolor* is given. Except for the presence of a nodule interpreted as a rudimentary paraseptal cartilage, conditions are very similar to those in more advanced snakes such as *Natrix*. The most striking differences from the saurian condition are shown by the morphology of the bony snout, especially the palate, and by certain features of the nasal capsule.

9. A comparison between *Varanus* and other lizards suggests that its isolated systematic position within the suborder Sauria is fully in accord with the findings described.

10. A comparison with snakes shows that, in spite of its peculiarities, the genus *Varanus* conforms in all essential respects to the saurian rather than to the ophidian pattern. On the evidence cited in this paper, and on a consideration of certain other cranial features, it is suggested that a close relationship between the Varanidae and the Serpentes cannot yet be regarded as firmly established.

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REFERENCES

Adams, W. E. (1942). Observations on the lacertilian sympathetic system. *J. Anat., Lond.*, **77**, 6–11.

Bäckström, K. (1931). Rekonstruktionsbilder zur Ontogenie des Kopfskeletts von *Tropidonotus natrix*. *Acta Zool., Stockh.*, **12**, 83–144.

Bahl, K. N. (1937). Skull of *Varanus monitor* (Linn). *Rec. Indian Mus.* **39**, 133–174.

Barge, J. A. J. (1937). Mundhöhlendach und Gaumen. In *Handbuch der Vergleichenden Anatomie der Wirbeltiere* (ed. L. Bolk), **3**. Berlin und Wien: Urban und Schwarzenberg.

Beecker, A. (1903). Vergleichende Stilistik der Nasenregion bei den Sauriern, Vögeln und Säugetieren. *Morph. Jb.* **31**, 565–619.

Bellairs, A. d'A. (1949). The anterior brain-case and interorbital septum of Sauropsida. *J. linn. Soc. (Zool.)*. In the Press.

Bellairs, A. d'A. & Boyd, J. D. (1947). The lachrymal apparatus in lizards and snakes. I. The brille, the orbital glands, lachrymal canaliculi and origin of the lachrymal duct. *Proc. zool. Soc. Lond.* **117**, 81–108.

Born, G. (1879). Die Nasenhöhlen und der Thränennasengang der amnioten Wirbelthiere. *Morph. Jb.* **5**, 62–140.

Bruner, H. L. (1907). On the cephalic veins and sinuses of reptiles, with a description of a mechanism for raising the venous blood-pressure in the head. *Amer. J. Anat.* **7**, 1–118.

Camp, C. L. (1923). Classification of the Lizards. *Bull. Amer. Mus. nat. Hist.* **48**, 289–482.
Camp, C. L. (1940). California mosasaurs. *Mem. Univ. Calif.* **13**, 1–68.
Corti, A. (1847). *De systemate vasorum Psammosauri grisei.* Vindobonae.
Cowles, R. B. (1930). The life history of *Varanus niloticus* (Linnaeus). *J. Ent. Zool.* **22**, 3–31.
de Beer, G. R. (1937). *The Development of the Vertebrate Skull.* Oxford University Press.
Fahrenholz, C. (1937). Drüsen der Mundhöhle. In *Handbuch der Vergleichenden Anatomie der Wirbeltiere* (ed. L. Bolk), **3**. Berlin und Wien: Urban und Schwarzenberg.
Fischer, J. G. (1852). Die Gehirnnerven der Saurier anatomisch untersucht. *Abh. Naturw. Hamburg*, **2**, 109–212.
Gaupp, E. (1888). Anatomische Untersuchungen über die Nervenversorgung der Mund- und Nasenhöhlendrüsen der Wirbelthiere. *Morph. Jb.* **14**, 436–489.
Gaupp, E. (1900). Das Chondrocranium von *Lacerta agilis*. Ein Beitrag zum Verständnis des Amniotenschädels. *Arb. anat. Inst., Wiesbaden* (*Anat. Hefte*), **15**, 435–592.
Hafferl, A. (1933). Das Arteriensystem. In *Handbuch der Vergleichenden Anatomie der Wirbeltiere* (ed. L. Bolk), **6**. Berlin und Wien: Urban und Schwarzenberg.
Haines, R. W. (1941). Epiphyseal structure in lizards and marsupials. *J. Anat., Lond.*, **75**, 282–294.
Haller vom Hallerstein, V. (1937). Zerebrospinales Nervensystem (Zentralnervensystem). In *Handbuch der Vergleichenden Anatomie der Wirbeltiere* (ed. L. Bolk), **2**. Berlin und Wien: Urban und Schwarzenberg.
Jarvik, E. (1942). On the Structure of the Snout of Crossopterygians and Lower Gnathostomes in General. *Zool. Bidr. Upsala*, **21**, 235–675.
Kesteven, H. L. (1940). The osteogenesis of the base of the Saurian cranium and a search for the parasphenoid bone. *Proc. Linn. Soc. N.S.W.* **66**, 447–467.
Lakjer, T. (1927). Studien über die Gaumenregion bei Sauriern im Vergleich mit Anamniern und primitiven Sauropsiden. *Zool. Jb.* (Anat. Abt.), **49**, 57–356.
Malan, M. E. (1941). The cranial anatomy of the genus *Gerrhosaurus*. *S. Afr. J. Sci.* **37**, 192–217.
Mertens, R. (1942). Die Familie der Warane (Varanidae). *Abh. senckenb. naturf. Ges.* **462**, 1–116; **465**, 117–234.
Noble, G. K. & Kumpf, K. F. (1936). The function of Jacobson's Organ in Lizards. *J. gen. Psychol.* **48**, 371–382.
O'Donoghue, C. H. (1920). The blood vascular system of the Tuatera, *Sphenodon punctatus*. *Philos. Trans.* B, **210**, 175–252.
Peyer, B. (1937). Zähne und Gebiss. In *Handbuch der Vergleichenden Anatomie der Wirbeltiere* (ed. L. Bolk), **3**. Berlin und Wien: Urban und Schwarzenberg.
Pratt, C. W. M. (1948). The morphology of the ethmoidal region of *Sphenodon* and lizards. *Proc. zool. Soc. Lond.* **118**, 171–201.
Rice, E. L. (1920). The development of the skull in the skink, *Eumeces quinquelineatus*. *J. Morph.* **34**, 120–216.
Romer, A. S. (1945). *Vertebrate Paleontology.* University of Chicago Press.
Watkinson, G. B. (1906). The cranial nerves of *Varanus bivittatus*. *Morph. Jb.* **35**, 450–472.
Wegner, R. N. (1922). Der Stützknochen, Os nariale, in der Nasenhöhle bei den Gürteltieren, Dasypodidae, und seine homologen Gebilde bei Amphibien, Reptilien und Monotremen. *Morph. Jb.* **51**, 413–492.
Willard, W. A. (1915). The cranial nerves of *Anolis carolinensis*. *Bull. Mus. comp. Zool. Harv.* **59**, 17–116.
Williston, S. W. (1925). *The Osteology of the Reptiles.* Harvard University Press.

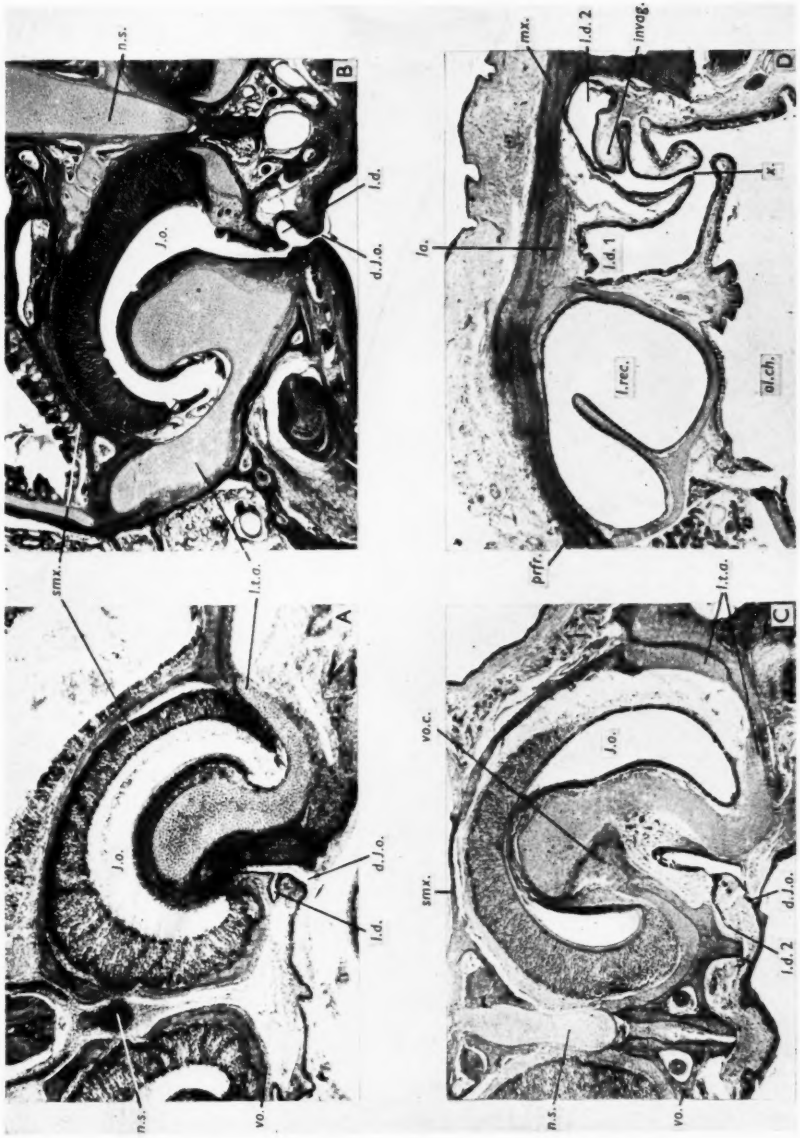
Abbreviations to Text-figures and Plate

a.ch.	anterior chamber of nasal sac.	*ch.*	choanal tube.
ad.co.	aditus conchae.	*ch.g.*	choanal groove.
add.m.	adductor mandibulae muscle.	*chor.*	choroid.
a.e.n.	anterior external nostril.	*c.J.o.*	cartilage of Jacobson's organ.
a.n.t.	anterior nasal tube.	*c.l.rec.*	capsule of lateral recess.
aosp.	antorbital space of nasal sac.	*co.*	nasal concha.
a.sp.	anterior space of nasal sac.	*co.mu.by.*	concavity for mushroom body.
br.	brille (spectacle).	*con.s.*	conjunctival space.
cart.	nodules of cartilage beneath anterior cupola.	*cor.*	cornea.
		co.z.	conchal zone of nasal sac.

cup. anterior cupola.
d.c.z. dorsal conchal zone.
d.Hd.gl. duct of Harderian gland.
d.J.o. duct of Jacobson's organ.
d.na.gl. duct of nasal gland.
d.n.p. ductus nasopharyngeus.
ec.c. ectochoanal cartilage.
ecpt. ectopterygoid.
eth.a. ethmoidal artery.
eth.g. ethmoidal ganglion.
f. flange of cartilage.
f.end.a. fenestra endonarina anterior.
f.endch. fenestra endochoanalis.
f.ex.a. fenestra exonarina anterior.
f.exch. fenestra exochoanalis.
f.l. fenestra in nasal septum.
f.olf. fenestra olfactoria.
fo.ap. foramen apicale.
fo.ep. foramen epiphaniale.
fr. frontal.
f.vo.ex. fenestra vomeronasalis externa.
f.vo.in. fenestra vomeronasalis interna.
gl. gland.
Hd.gl. Harderian gland.
hy.c. hypochoanal cartilage.
i.ch. inner choana.
invag. invagination of wall of lachrymal duct 2 in *Varanus*.
i.ob. inferior oblique muscle.
i.o.s. interorbital septum.
J.o. Jacobson's organ.
ju. jugal.
la. lachrymal.
l.c. lachrymal canaliculus.
l.d. lachrymal duct.
l.d. 1, *l.d.* 2. lachrymal ducts 1 and 2 in *Varanus*.
l.p. 1, *l.p.* 2. lachrymal puncta 1 and 2.
l.rec. lateral recess of nasal sac.
l.t.a. lamina transversalis anterior.
l.t.a.' free posterior end of *l.t.a.*
mu.by. mushroom body.
mx. maxilla.
mx.' palatal process of *mx.*
mx.a. maxillary artery.
na. nasal.
na.gl. nasal gland.
n.s. nasal septum.
o.ch. outer choana.
o.d.J.o. opening of duct of *J.o.*
ol.b. olfactory bulb.
ol.ch. olfactory chamber of nasal sac.
o.l.d. 1, *o.l.d.* 2. openings of lachrymal ducts 1 and 2.
ol. ep. olfactory epithelium.
o.s. orbital sinus.
pal. palatine.
pal.a. palatine artery.
p.al.in. processus alaris inferior.
p.al.su. processus alaris superior.
pas. parasphenoid.
p.gl. palatine gland.
pmx. premaxilla.
p.mx.a. processus maxillaris anterior.
p.mx.p. processus maxillaris posterior.
pmx.b. premaxillary bar.
pn.c. paranasal cartilage.
pn.c.' separated part of *pn.c.*
pn.w. postnasal wall (planum antorbitale).
p.pmx. palatal process of *pmx.*
prfr. prefrontal.
ps.c. paraseptal cartilage.
ps.c.' fragmented part of *ps.c.*
pt. pterygoid.
pt.c. parietotectal cartilage.
r.na.c. roof of nasal capsule.
re.ep. respiratory epithelium.
ros.p. rostral process.
s.b.s. sub-brillar space (closed conjunctival space).
smx. septomaxilla.
so. supraorbital.
sph.c. sphenethmoid commissure.
s.t. spongy tissue round anterior chamber of nasal sac.
t. tooth and dental lamina.
to. tongue.
tr. trabecula communis.
v.co., *vo.c.* vomerine concha.
v.c.z. ventral conchal zone of nasal sac.
vo. vomer.
vo.cu. vomerine cushion.
vo.gl. vomerine gland.
vo.pmx. vomerine process of *pmx.*
x. connexion between *l.d.* 2 and cavity of nasal sac in *Varanus*.
I. olfactory nerve.
I'. vomeronasal nerve.
V*a*. ophthalmic and ethmoidal nerve.
V*a.m.* medial ethmoidal branch of V*a*.
V*a.l.* lateral ethmoidal branch of V*a*.
V*b*. maxillary nerve.
VII*p*. palatine branch of facial nerve.

EXPLANATION OF PLATE

A. *Xenopeltis unicolor*, subadult. Transverse section through right organ of Jacobson showing relationship of lachrymal duct to duct of Jacobson's organ. (*c.* × 260.) B. *Tupinambis teguixin*, subadult. Transverse section through left organ of Jacobson. (*c.* × 20.) C. *Varanus monitor*, subadult. Transverse section through right organ of Jacobson. The relationship of the lachrymal duct to Jacobson's organ is similar in A, B and C. (*c.* × 36.) D. *Varanus monitor*, subadult. Transverse section through side of snout showing ending of lachrymal duct 1, and connexion (*x*) between lachrymal duct 2, and cavity of nasal sac. (*c.* × 36.)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A CYTOARCHITECTURAL INVESTIGATION INTO THE BOUNDARIES OF CORTICAL AREAS 13 AND 14 IN THE HUMAN BRAIN

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INTRODUCTION

Walker (1940) has described two new areas in the posterior orbital surface of the macaque brain: area 13 in the posterior orbital gyrus and area 14 in the gyrus rectus beneath and medial to the olfactory tract. Both areas were essentially agranular. No corresponding investigations have been reported in the human brain, nor did Walker fully discuss the relationship of his two new areas to previously described cortical subdivisions in man. Von Bonin (1944), however, tried to correlate almost agranular zones in the posterior orbital surface of the human brain, described by von Economo & Koskinas (1925), with an 'area orbitalis agranularis' homologous in all mammals so far investigated. As will become apparent, however, this correlation does not seem to cover entirely the more complex conditions which appear to obtain in man. In the light of these considerations, and since the neurophysiology of this region has lately aroused much interest, re-examination of its cytoarchitecture seemed to be a matter of some urgency.

Bailey & Sweet (1940) stimulated the gyrus orbitalis posterior in monkeys and cats causing inhibition of respiration, rise of blood pressure and decrease in the tonus of the gastric musculature. Ruch & Shenkin (1943) ablated area 13 in a series of monkeys producing a marked degree of hyperactivity. Fulton (1947) mentioned that experiments carried out jointly with Livingston confirmed the results of Ruch & Shenkin in monkeys as far as area 13 was concerned. Small bilateral lesions of area 13 in one chimpanzee, however, did not cause hypermotility, from which Fulton concluded that the region may be larger than in the monkey and in man may be larger still. Ward & McCulloch (1947) recorded neuronographic evidence of strong projections in monkeys from posterior and medial portions of the orbital surface (areas 10, 47, 53 in Bailey's terminology) to the paraventricular nucleus of the hypothalamus and from area 47 and posterior area 45 to the posterior hypothalamic area. More recently, Delgado & Livingston (1948) published their results obtained by electrical excitation on monkeys and dogs, which showed that the active region concerned with respiratory and blood pressure changes coincides with area 13 and that in some animals its limits are sharp. After bilateral ablation of area 13 their monkeys showed elevated temperatures in the hind limbs.

That this region in man seems to be concerned with similar functions, has been shown in a paper by Meyer & McLardy (1948), dealing with posterior cuts in prefrontal leucotomy; nine of their cases, with bilateral involvement of the central third

of the posterior half of the orbital region (liable to include the region corresponding to area 13 in monkeys), developed post-operative disturbances, many of which seemed to have an autonomic basis.

Although the precise delineation of the agranular orbital cortex in man was the object of this study, it has been necessary, for the understanding of the correct boundaries, to include a cytoarchitectonic description of adjacent areas.

MATERIAL AND METHODS

The cytoarchitectonic investigation of the orbital surface was carried out on nine hemispheres taken from seven different cases. Case 1, a normal male aged 40, was fully investigated in serial sections through the whole length of the left orbital region. Case 2, a normal male aged 20, was investigated in serial sections through the posterior half of the left orbital region, and through a sample block from the anterior half. Cases 3–7 were examined in serial sections through sample blocks from three different levels, chosen within the anterior, middle and posterior thirds of the orbital surface. This material (cases 3–7) was taken from four patients in whom prefrontal leucotomy had been performed without damaging the orbital region or its projections, and from one patient with an old arteriosclerotic softening around the Sylvian fissure.

The material, which was obtained approximately 24 hr. after death, was fixed in 10 % formalin and subsequently embedded in celloidin. Serial sections (30 μ) were cut, of which every twenty-fifth was stained by the Nissl method. In these comparatively thick sections all the peculiarities of each region were emphasized, without obscuring finer details. Some myelin-stained sections (Heidenhain's modification of Weigert's method) were also examined to exclude the presence of any small lesion not obvious in the Nissl preparations. No lesions were found.

Owing to the convolutional characteristics of the orbital region, i.e. arrangement of sulci and gyri more or less parallel to the medial and lateral surfaces (Text-fig. 1*b*), only coronal sections were used. In this way the desired 'ideal plane' at right angles to the sulci and cortical surface was obtained and distortion of the cortical pattern due to obliquity of section was avoided.

The criteria employed for differentiation of the various areas were chosen with the greatest care, in order to avoid the criticism brought forward by Lashley & Clark (1946). Only striking alterations in the laminar pattern were regarded as a basis for subdivision and the characteristics of each area, as described below, could be recognized throughout the nine hemispheres. Minor differences of cell size, cell shape, or width of cortex, which could be interpreted as individual differences were noted, but never by themselves taken as an indication of a distinct cortical field. The above applies mainly to the criteria employed for parcellation in the medio-lateral axis; antero-posteriorly the subdivision was based on the presence or absence of an internal granular layer, a criterion which is valid beyond doubt.

It proved extremely difficult to carry out parcellation by microscopical observation only. Therefore, in case 1 photomicrographs of every hundredth section of the gyrus rectus, of the orbital gyri and of the pars orbitalis of the inferior frontal gyrus were taken and were fixed to a board in their correct topographical position. In this way a reasonably representative sample of the microscopic structure of the entire

orbital surface could be viewed simultaneously; an invaluable help for parcellation. In addition, the surface outline of these sections was drawn by projection and the boundaries of the architectonic areas marked in under microscopic control. The chart (Text-fig. 1*a*), representing a reconstruction of the orbital surface of case 1, was based on these drawings. The level of each section has been shown by its corresponding serial number at the side of the chart; thus the exact location of photomicrographs (Pls. 1–3) can be easily determined. Where wide transitional zones between two areas occurred they have been indicated, using the symbols of both areas concerned simultaneously. The next charts (Text-fig. 2*a*,*b*) show the position of Brodmann's (1914, 1925) and von Economo & Koskinas' (1925) areas as they would appear, if projected on to the orbital surface of case 1. The thick lines in von Economo & Koskinas' chart indicate the location of their photographs.

Following Walker's example Brodmann's numerical nomenclature has been used as far as possible throughout the region investigated. It proved necessary, however, to introduce certain further terms: i.e. area recta anterior and posterior and area 47 anterior and posterior.* Reference to the most posterior extension of the orbital cortex—transition into insular and parolfactory regions—has been made in von Economo & Koskinas' terms *FJ* and *FL*, since, except for area 25, no terminology of Brodmann's is available. It seems likely that in future investigations von Economo & Koskinas' terminology will have to be introduced throughout as being more logical and more flexible.

RESULTS

The following areas have been recognized throughout the nine hemispheres; their position can be located from Text-fig. 1*a*.

Area recta anterior and area recta posterior (Text-fig. 1*a*)

Cytoarchitecture. Area recta anterior lies on the anterior half of gyrus rectus expanding laterally to cover the most medial part of the adjacent orbital gyrus. Anteriorly it terminates with the end of the olfactory sulcus; the subrostral sulcus forms its border on the medial surface.

Its main characteristics are: narrowness of the cortex as a whole; especially accentuated narrowness in layer III; cells of equal size throughout III; distinctly marked, though narrow internal granular layer and—in places—arrangement of the infragranular layers in vertical columns. None of the laminae is sufficiently well marked to give rise to horizontal striation (Pl. 1, fig. 1).

Area recta posterior covers the posterior half of gyrus rectus, and extends laterally into the most medial part of the adjacent orbital gyrus. Anteriorly it is bordered by area recta anterior, posteriorly it gradually merges into the parolfactory area of Broca (*FL* of von Economo & Koskinas). The sulcus subrostralis forms its boundary on the medial surface.

Area recta posterior may best be described as an agranular subdivision of area recta anterior, for it retains all its other characteristics (Pl. 1, fig. 2). The change from granular to agranular cortex is not a sudden one, but takes place over a fairly

* Brodmann already suggested a possible further subdivision of his area 11 into a medial area recta and a lateral area orbitalis interna on the strength of different cytoarchitecture. But he never actually executed this suggestion by drawing these areas separately on his chart.

extensive transitional zone in which the internal granular layer becomes gradually narrower, less dense and more intermingled with large cells from layers III and V. No difference in cortical structure could be noted between the crown of gyrus rectus and the walls of the olfactory sulcus.

Discussion. The position and structure of area recta anterior and area recta posterior—as described in this paper—seem to be identical with von Economo & Koskinas' description of their areas *FG* (their plate XXXV) and the orbital portion of *FH*, respectively.

Brodmann's* area 11 includes in its most medial portion the site of our areas recta anterior and posterior. Its architecture, however, described as being uniform for the entire area, resembles only the structure in our transitional zone, between areas recta anterior and posterior.

A cytoarchitectonic parcellation of gyrus rectus was undertaken by Ngowyang (1932), who confirms on the whole Vogt's (1910) myeloarchitectonic subdivisions. Ngowyang distinguishes between anterior granular and posterior dysgranular portions, the former being roughly identical with area recta anterior of the present writer. Only a small area in the depths of the olfactory sulcus is described as quite agranular but is assigned to the parolfactory region.

Walker, in the macaque, did not describe any area comparable to our area recta anterior. His area 14, however, occupies a position apparently similar to our area recta posterior; but the description and illustration of its architecture—taken from the walls of the olfactory sulcus—show it to be much more akin to the parolfactory cortex (*FL*) in man, although the latter is restricted to about the posterior tenth of gyrus rectus. It therefore seems probable that his area 14 is not homologous with our area recta posterior, a view which finds further support from von Bonin & Bailey (1947) who, adopting von Economo's terminology for their map of *Macaca mulatta*, assign the entire area equivalent to Walker's 14 to parolfactory *FL*.†

Areas 11 *and* 13 (Text-fig. 1*a*)

Cytoarchitecture. Area 11 represents the cortex of the anterior two-thirds of the medial orbital gyrus, forming the lateral boundary of area recta anterior. It curves round the anterior end of the olfactory sulcus, extending slightly on to the medial aspect of the hemisphere. Laterally it encroaches upon the middle orbital gyrus.

Its cytoarchitecture is similar to area recta anterior, the main differences being: gradual medio-lateral increase in total width, evenly distributed throughout the layers; a denser layer III with larger cells in its depths, which permit its subdivision into III*a* and III*b*; a more pronounced layer V*a* and layer VI*a* with lighter layer V*b*,

* As the data on which Brodmann (1908, 1910, 1912, 1914 and 1925) based his cytoarchitectonic map of the human brain have never been published, Rose's (1935) detailed histological descriptions and photographs of Brodmann's areas have been used for the assessment of the architectonic characteristics.

† As far as can be seen from the literature no autonomic responses have so far been obtained from Walker's area 14 in animals. On the other hand, Fulton (1947) describes a human case in whom during the course of an operation for a pituitary tumour the most posterior portion of, what appears to be from his description, gyrus rectus was accidentally fixed bilaterally with Zenker's fluid. (This region would most likely include parolfactory as well as cortex from the posterior part of our area recta posterior.) The patient developed a condition similar to sham-rage in animals.

giving rise to distinct horizontal striation; and no columnar arrangement of the infragranular layers (Pl. 2, fig. 1).

Area 13 covers the posterior third of the medial orbital gyrus, or the posterior fifth only, if the transitional zone be excluded. Anteriorly it is bordered by area 11, posteriorly by von Economo & Koskinas' area 'fronto-insularis' (*FJ*) in the gyrus transversus insulae.

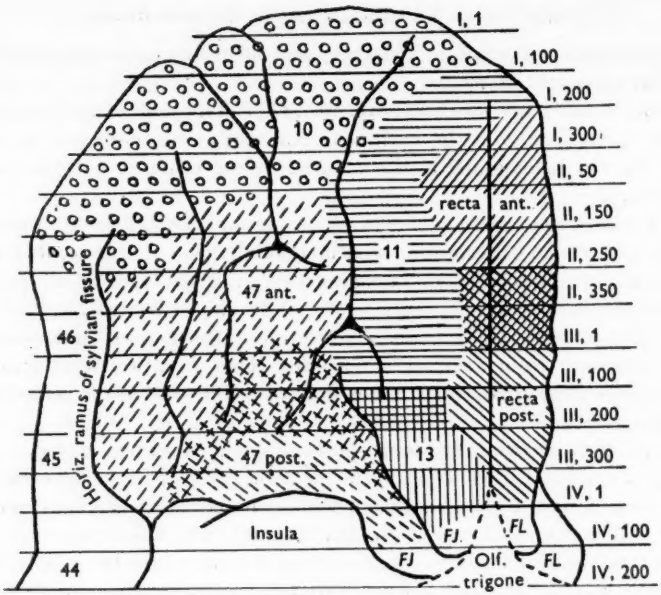
Just as I regard area recta posterior as an agranular subdivision of area recta anterior, so area 13 can be termed an agranular subdivision of area 11. It retains all the main characteristics of area 11, including a very marked layer V*a* with especially large cells towards the posterior end and a well-developed layer VI*a* (Pl. 2, fig. 2). The latter two features give rise to distinct horizontal striation, which also constitutes the main difference from area recta posterior. The transition from granular to agranular cortex is gradual, but extends only over 100 sections as compared with 200 on gyrus rectus.

Discussion. Neither Brodmann nor von Economo & Koskinas describe any area which is topographically or structurally identical with areas 11 and 13, as just defined. Topographically these areas fall within the lateral portion of Brodmann's area 11—i.e. his suggested area 'orbitalis interna'—for which, however, neither a separate structural description was given nor a further possible subdivision into granular and agranular parts considered.* With regard to von Economo & Koskinas' chart our areas 11 and 13 fall within the medial portion of their area *FF*. No part, however, of *FF* is described as similar to our 11 and 13, on the contrary representative photographs of the anterior granular and posterior agranular subdivisions (*FFg* and *FFa*), both taken from lateral portions, are identical in every detail with those of our areas 47 anterior and posterior, to be described later (Pl. 3, figs. 1 and 2). A comparison, however, of Pl. 2, figs. 1 and 2 with Pl. 3, figs. 1 and 2 will show the striking difference between our areas 11 and 13 on the one hand, and our 47 anterior and posterior on the other. Separation of areas 11 and 13 from the field *FF* seems therefore appropriate, and finds further support from Vogt's (1910) and Strasburger's (1937) description of the myeloarchitecture of this region; i.e. 'unistriär' corresponding to our areas 11 and 13, but 'bistriär' and 'unitostriär' to our areas 47 anterior and posterior, respectively.

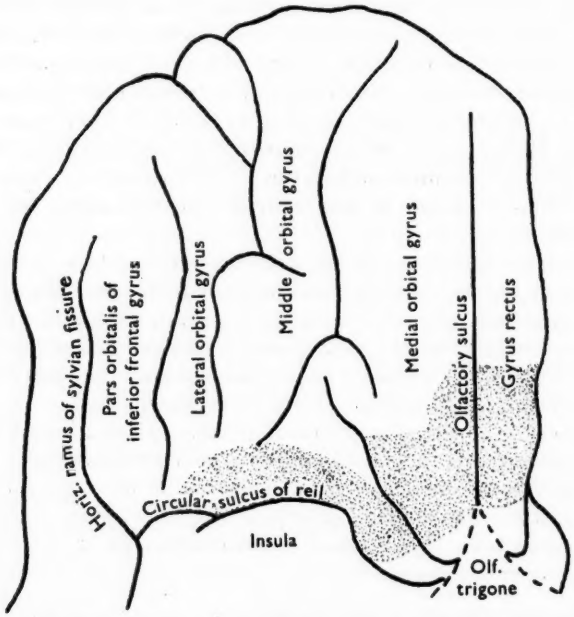
Walker's description and photographs of his areas 11 and 13 in the macaque show them to be homologous in topography and structure to those described in man in this paper. There exists only the difference in expanse, transition from areas 11 to 13 in the macaque occurring between the anterior and posterior half of the orbital gyrus, whilst in man it takes place between the middle and posterior third of the (medial) orbital gyrus. Thus area 13 in man is far more restricted.

In their charts of the macaque von Bonin (1944) and von Bonin & Bailey (1947) homologize Walker's area 13 with their areas 47 and *FF* respectively. It is not my intention to go into the often very contradictory evidence of homologies; in man, however, it must be stressed that investigation seems to show more complicated conditions, area 13 being by no means identical with area 47, even in the latter's most posterior agranular portion.

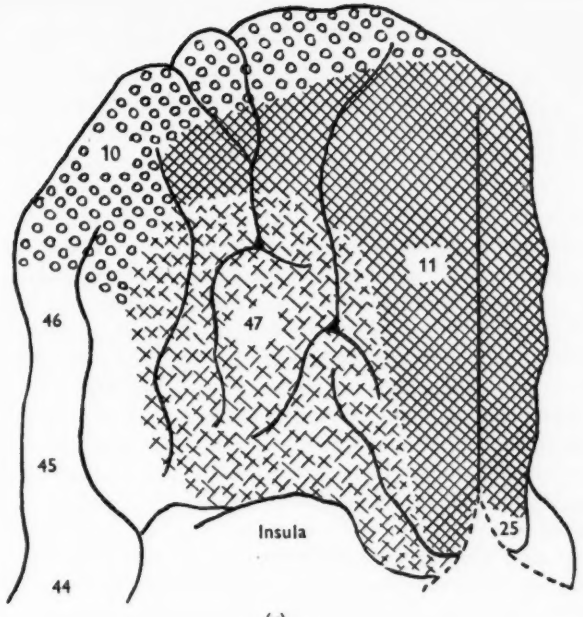
* Brodmann's original term 'area 11' has none the less been retained in this study on account of its customary usage in man and its homology to Walker's area 11 in the macaque.



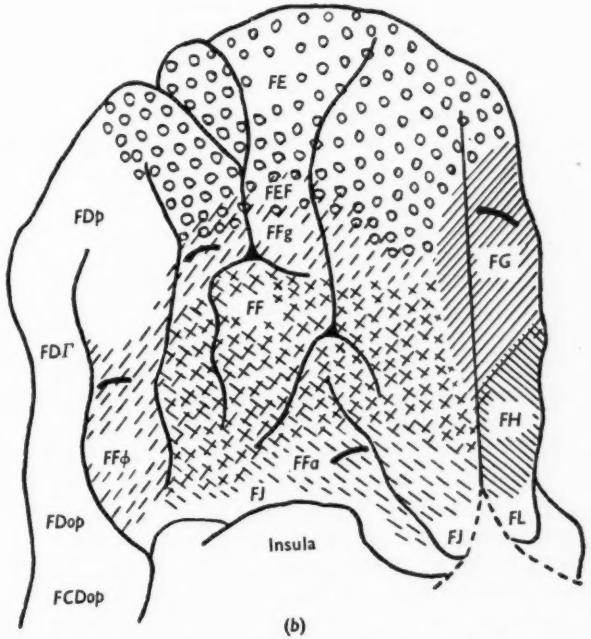
Text-fig. 1*a*. Parcellation of the orbital cortex in case 1, left; temporal pole removed. Reconstruction from diagrams of every hundredth section at a magnification of two (allowing 6 mm. for hundred sections of 30μ thickness). The curvature of the brain has been flattened out.



Text-fig. 1*b*. Diagram indicating the total amount of agranular orbital cortex (stippled area), and the terminology used for gyri and sulci.



(*a*)



(*b*)

Text-fig. 2. Brodmann's (*a*) and von Economo & Koskinas' (*b*) areas projected upon the orbital surface of case 1. The markings have been adapted to those of case 1, i.e. equal markings signify identically described cytoarchitecture.

Areas 47 anterior and posterior (Text-fig. 1*a*)

Cytoarchitecture. Area 47 anterior lies on the orbital portion of the inferior frontal convolution and extends medially to cover the lateral and middle orbital gyri. Anteriorly it is limited by the polar cortex (area 10); posteriorly by its agranular subdivision, area 47 posterior. The horizontal ramus of the Sylvian fissure provides its approximate lateral boundary, the medial orbital sulcus its medial one.

Area 47 anterior is differentiated from area 11, which it borders laterally, by a considerable increase in width and density of the cortex, especially marked in layers IV and VI*a*, the latter being far denser than in any of the previously described areas. The fifth layer also becomes wider but very light and cell-poor, especially V*b*. This produces a very pronounced horizontal striation. There is also a tendency to arrangement of the cells in vertical columns (Pl. 3, fig. 1).

Area 47 posterior is a small zone which lies in the most posterior end of the lateral and middle orbital gyri. Anteriorly it is bordered by area 47 anterior, posteriorly by the anterior portion of the circular sulcus of Reil and von Economo & Koskinas' area fronto-insularis (*FJ*).

Area 47 posterior can best be regarded as an agranular subdivision of area 47 anterior whose main characteristics, i.e. emptiness of layer V and dense, well-developed layer VI*a*, are preserved. Horizontal striation, however, becomes indistinct owing to the lack of an internal granular layer; the arrangement in vertical columns becomes less marked; and the transition from fifth to sixth layer is in places difficult to assess (Pl. 3, fig. 2). In addition, the cortex becomes less dense and the individual cells larger as one progresses posteriorly. The increase in cell size applies mainly to layer V*a*, where indeed very large cells can be seen occasionally, nowhere, however, giving rise to distinct striation. This latter feature constitutes one of the main differences from area 13, which forms its medial boundary. The division of area 47 into granular and agranular cortex is less distinct than in previously described regions, the transitional zone—in some cases—being a much wider one, thus confining the completely agranular cortex to a narrow posterior strip in the lateral and middle orbital gyri. The cortex of the orbital portion of the inferior frontal convolution remains granular throughout its antero-posterior length.

Discussion. There seems to exist considerable confusion in the literature with regard to the topography and structure of area 47, which may be attributable to its apparently great variability. Brodmann's area 47—forming part of the 'infrafrontal region' in his revised terminology—occupies a similar topographical position to our areas 47 anterior and posterior, but does not reach the horizontal ramus of the Sylvian fissure at any point (the granular cortex of this region being assigned to his areas 45 and 46). The description of its architecture, however, with a feebly developed internal granular layer and wide infragranular layers renders it akin only to our transitional zone between areas 47 anterior and posterior.

Von Economo & Koskinas would include the site of our areas 47 anterior and posterior in the lateral two-thirds of their area *FF*, giving, in contradistinction to Brodmann, the horizontal ramus of the Sylvian fissure as its lateral boundary. They stress the fact that this region has no uniform character and that it is therefore impossible to give a description of its architecture equally applicable to every part.

They mention and illustrate besides an anterior granular portion, *FFg*, a posterior agranular *FFa* and a lateral granular *FF*ϕ. Apart from these they seem to have encountered considerable individual differences, *FF* showing in extreme cases a wide, dense internal granular layer throughout. Their representative photographs of *FFg* and *FFa*, both taken from lateral portions (their plates XXXII and XXXIII), are, as already mentioned, in every detail identical with those of our areas 47 anterior and posterior, respectively. A region corresponding in structure to *FF*ϕ could also be clearly seen in the present material.

Kreht (1936*a*) studied the cytoarchitecture of the region equivalent to our areas 47 anterior and posterior, using Vogt's (1910) terminology. His results with regard to anterior 'granularity' and posterior 'dysgranularity' are essentially in accordance with the findings in this study.

Walker, in the macaque, describes no area 47, nor any area homologous to our areas 47 anterior and posterior. The orbital portion of his area 12, which occupies a similar position to our area 47, displays a very different cytoarchitecture, and his area 13 has been shown in the present investigation to be homologous to area 13 in man.

Von Bonin (1944) emphasizes the presence of a homologous area 47 ('orbitalis agranularis'), in all mammals so far investigated (including man), and identifies it with Walker's area 13 in the macaque. In his joint publication with Bailey (1947) he terms it *FF* and gives a description of its architecture very similar indeed to that of Walker's 13. In his publication on Galago (1945), however, he describes area 47 as displaying a light fifth and a dense sixth layer, indicating a certain structural relationship to our area 47 posterior. The question of homology must therefore remain open.

Some difficulties were encountered in delimiting area 47 anterior from area 10. For this reason no transitional zone has been drawn in on the chart (Text-fig. 1*a*) and the boundary given should be regarded only as a rough indication.

Individual differences

The fact that the main characteristics of each region could be recognized in all nine hemispheres by no means excludes the presence of distinct individual differences. Width of cortex, cell size, structure and density and above all granularity varied greatly from case to case. It therefore seems possible that the discrepancies between the various findings of different observers may be largely due to their considering such individual differences too much as a basis for parcellation, as indeed has been pointed out by Lashley & Clark (1946). Whether individual differences also exist with regard to the extent of granular and agranular orbital cortex in antero-posterior direction, or with regard to the size of the architectonic areas as a whole—such as Kreht (1936*b*) actually demonstrated for the 'wider area of Broca'—cannot be established from the present material. This question will have to be investigated later in serial sections throughout the length of the orbital surface in a large number of brains.

CONCLUSIONS AND SUMMARY

The most striking fact brought out by this study is that in all the cases a granular area recta anterior and area 11 could be subdivided from an agranular area recta posterior and area 13. The difference between a granular and an agranular area 47 was not so distinct, because in some cases the transitional zone was wide and complete agranularity was reached only in the most posterior levels. The cortex on the orbital portion of the inferior frontal gyrus was granular throughout its entire length in all cases.

The new terms, area recta anterior and posterior and area 47 anterior and posterior, have been introduced to signify the division into granular and agranular cortex.

Homology of Walker's area 13 in the macaque to area 13 in man could be established, while homology of his area 14 in the macaque to area recta posterior in man seemed extremely doubtful. A different terminology, i.e. area recta posterior, was therefore chosen for the latter.

As compared with Walker's (1940) findings in the macaque the agranular portion of the orbital cortex in man seems to be proportionately much smaller. It is difficult to say where the exact anterior boundary should be drawn, as all these areas show a comparatively wide transitional zone with gradually decreasing internal granular layer in antero-posterior direction. This problem, however, might be solved if a more accurate functional boundary were determined. Meanwhile, it is interesting to note that this region belongs to the agranular type of cortex and is therefore more akin to premotor (area 6) than to prefrontal cortex.

I wish to express my gratitude to Dr A. Meyer, at whose instigation this study was undertaken, for his criticism throughout.

REFERENCES

BAILEY, P. & SWEET, W. H. (1940). Effects on respiration, blood pressure and gastric motility of stimulation of orbital surface of frontal lobe. *J. Neurophysiol.* **3**, 276–281.

BONIN, G. VON (1944). Architecture of the precentral motor cortex and some adjacent areas. *Illinois Monographs in the Medical Sciences.* Vol. IV, nos. 1–4, pp. 7–82. Urbana: University of Illinois Press.

BONIN, G. VON (1945). The cortex of Galago. *Illinois Monographs in the Medical Sciences.* Vol. V, no. 3, pp. 5–55. Urbana: University of Illinois Press.

BONIN, G. VON & BAILEY, P. (1947). The Neocortex of *Macaca mulatta. Illinois Monographs in the Medical Sciences.* Vol. V, no. 4, pp. 1–163. Urbana: University of Illinois Press.

BRODMANN, K. (1908). Beiträge zur histologischen Lokalisation der Grosshirnrinde. VI. Mitteilung. Die Cortexgliederung des Menschen. *J. Psychol. Neurol., Lpz.*, **10**, 231–246.

BRODMANN, K. (1910). Feinere Anatomie des Grosshirns, in Lewandowsky, *Handbuch der Neurologie.* Vol. I, pt. 1, pp. 206–305. Berlin: J. Springer.

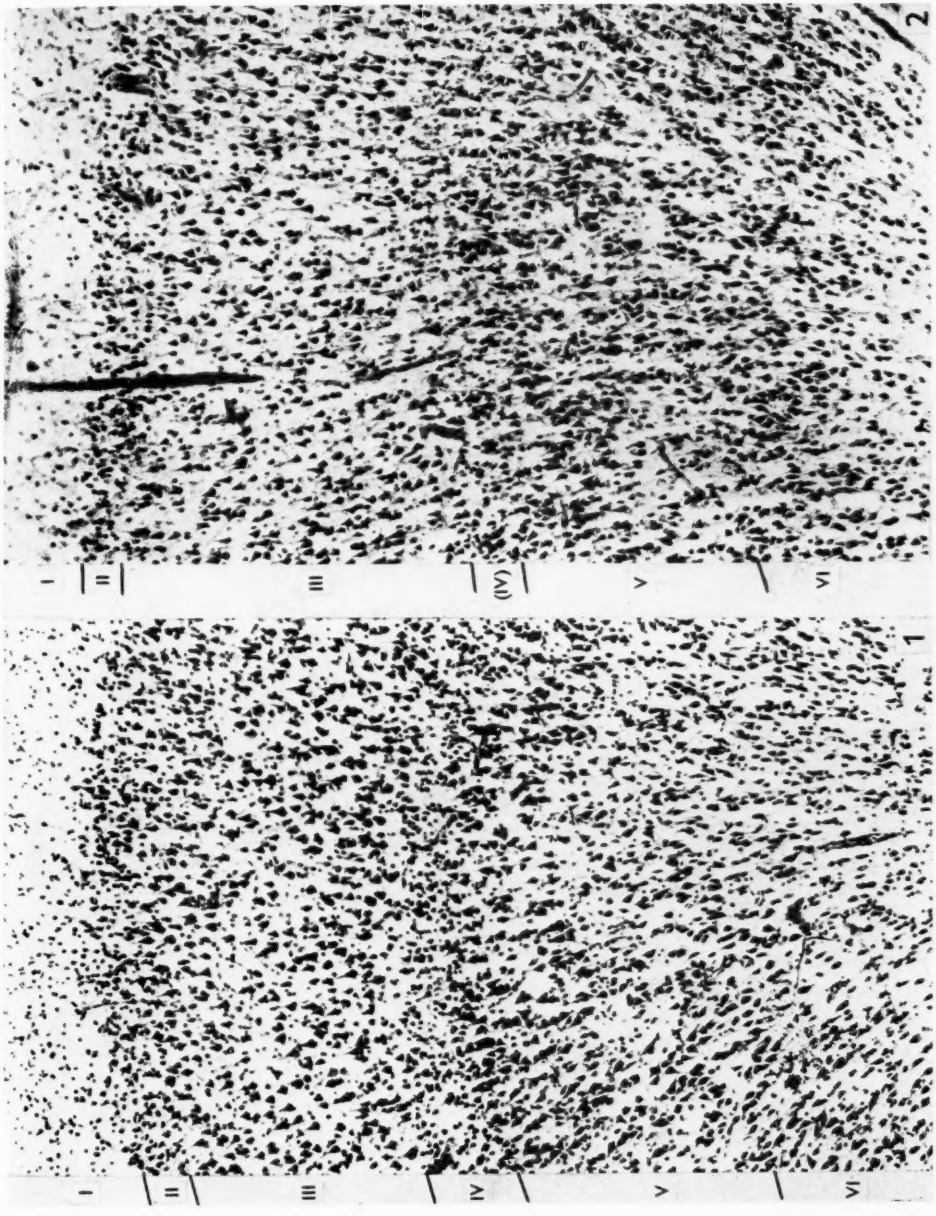
BRODMANN, K (1912). Neue Ergebnisse über die vergleichende Lokalisation der Grosshirnrinde mit besonderer Berücksichtigung des Stirnhirns. *Anat. Anz.* **41**, 157–216.

BRODMANN, K. (1914). Physiologie des Gehirns, in Bruns, *Neue Deutsche Chirurgie.* Vol. II, pt. 1, pp. 85–426. Stuttgart: F. Enke.

BRODMANN, K. (1925). *Vergleichende Lokalisationslehre der Grosshirnrinde,* pp. xii + 324. Leipzig: J. A. Barth.

DELGADO, J. M. R. & LIVINGSTON, R. B. (1948). Some respiratory, vascular and thermal responses to stimulation of orbital surface of frontal lobe. *J. Neurophysiol.* **11**, 39–55.

ECONOMO, C. VON & KOSKINAS, G. N. (1925). *Die Cytoarchitektonik der Hirnrinde des erwachsenen Menschen,* pp. xxxix + 811, Atlas. Berlin: J. Springer.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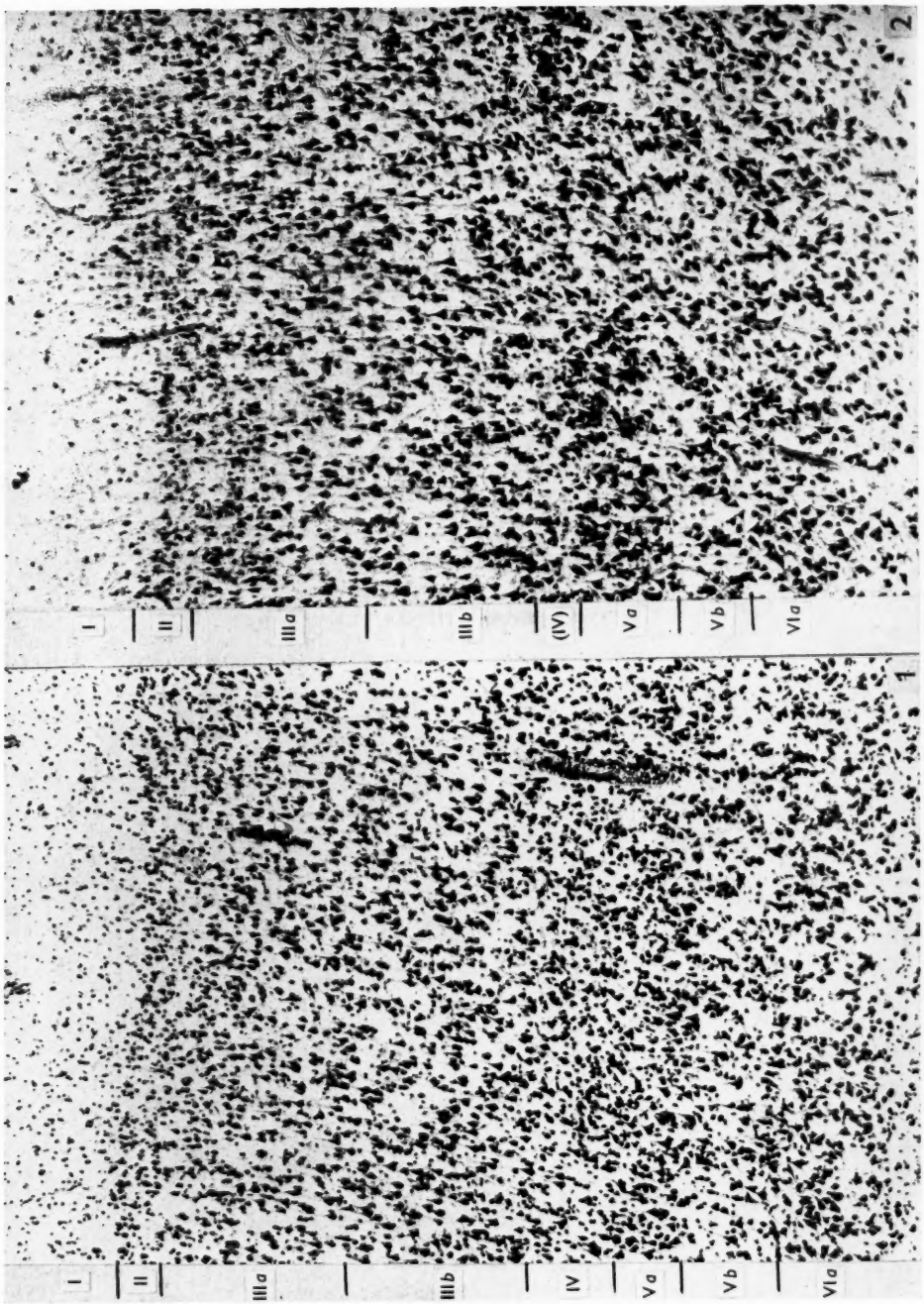
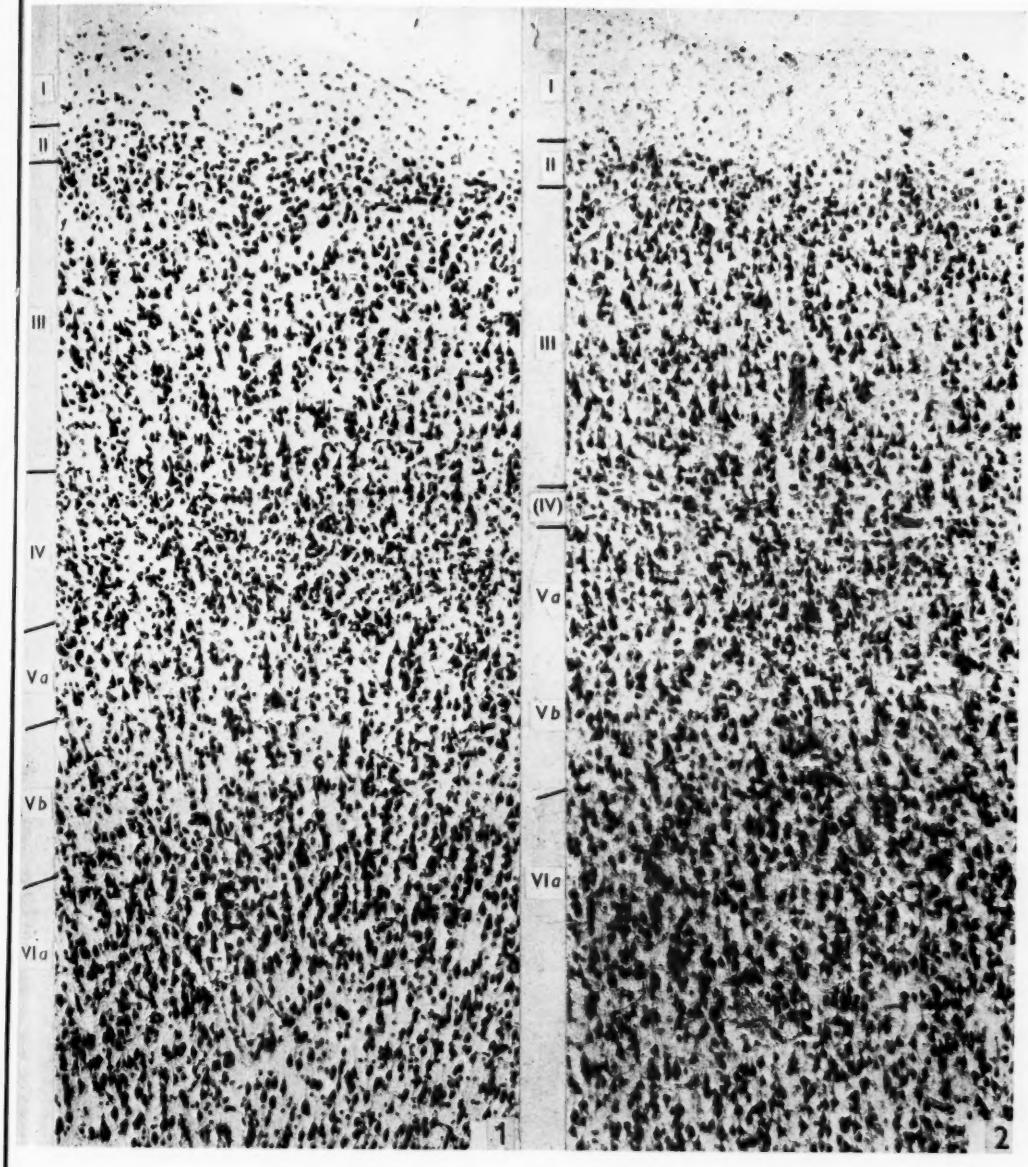


BECK—Cortical areas 13 and 14 in the human brain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FULTON, J. F. (1947). Physiological basis of frontal lobotomy. *Acta med. scand. Suppl.* **196**, 617–625.

KREHT, H. (1936*a*). Cytoarchitektonik und motorisches Sprachzentrum. *Z. mikr.-anat. Forsch.* **39**, 331–354.

KREHT, H. (1936*b*). Zur Volumengrösse der architektonischen Felder 55–66 einiger menschlicher Gehirne im Vergleich zu der des Schimpansen und Orang-Utan. *Z. mikr.-anat. Forsch.* **39**, 409–414.

LASHLEY, K. S. & CLARK, G. (1946). The cytoarchitecture of the cerebral cortex of Ateles: A critical examination of architectonic studies. *J. comp. Neurol.* **85**, 223–305.

MEYER, A. & MCLARDY, T. (1948). Posterior cuts in prefrontal leucotomy: A clinico-pathological study. *J. ment. Sci.* **94**, 555–564.

NGOWYANG, G. (1932). Die Zytoarchitektonik der Felder des gyrus rectus. *J. Psychol. Neurol., Lpz.*, **44**, 475–493.

ROSE, M. (1935). Cytoarchitektonik und Myeloarchitektonik der Grosshirnrinde, in Bumke-Foerster, *Handbuch der Neurologie*. Vol. I, pp. 588–788. Berlin: J. Springer.

RUCH, T. C. & SHENKIN, H. A. (1943). The relation of area 13 on the orbital surface of the frontal lobes to hyperactivity and hyperphagia in monkeys. *J. Neurophysiol.* **6**, 349–360.

STRASBURGER, E. H. (1937). Die myeloarchitektonische Gliederung des Stirnhirns beim Menschen und Schimpansen. *J. Psychol. Neurol., Lpz.*, **47**, 461–491 and 565–606.

VOGT, O. (1910). Die myeloarchitektonische Felderung des menschlichen Stirnhirns. *J. Psychol. Neurol., Lpz.*, **15**, 221–232.

WALKER, A. E. (1940). A cytoarchitectural study of the prefrontal area of the macaque monkey. *J. comp. Neurol.* **73**, 59–86.

WARD, A. A. JNR. & MCCULLOCH, W. S. (1947). The projection of the frontal lobe on the hypothalamus. *J. Neurophysiol.* **10**, 309–314.

EXPLANATION OF PLATES

All the photomicrographs are taken from Nissl preparations at a magnification of ×85. The '(IV)' in Pls. 1, 2 and 3, fig. 2, which illustrate agranular cortex, indicates the position of the outer stripe of Baillarger as seen in myelin-stained sections.

PLATE 1

Fig. 1. Case 1, left. Bl. II, 150. Photomicrograph of area recta anterior, on the crown of gyrus rectus.
Fig. 2. Case 1, left. Bl. III, 200. Photomicrograph of area recta posterior, on the crown of gyrus rectus.

PLATE 2

Fig. 1. Case 1, left. Bl. II, 150. Photomicrograph of area 11, in the centre of the medial orbital gyrus.
Fig. 2. Case 1, left. Bl. III, 275. Photomicrograph of area 13, in the centre of the medial orbital gyrus.

PLATE 3

Fig. 1. Case 1, left. Bl. II, 150. Photomicrograph of area 47 anterior, on the crown of the lateral orbital gyrus.
Fig. 2. Case 1, left. Bl. III, 275. Photomicrograph of area 47 posterior, in the centre of the middle orbital gyrus.

BONES, MUSCLES AND VITAMIN C

I. THE EFFECT OF A PARTIAL DEFICIENCY OF VITAMIN C ON THE REPAIR OF BONE AND MUSCLE IN GUINEA-PIGS*

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INTRODUCTION

The observations described in this paper, some of which have been mentioned in earlier communications (Kodicek & Murray, 1943; Murray & Kodicek, 1946), were made in the course of an investigation, which had negative results, of the alleged reopening of old healed fractures in prolonged vitamin C insufficiency. We found no evidence of reopening of healed fractures under these conditions, but we made certain other observations, the first three of which are the subjects of the present paper. These observations concerned (1) the repair of the fractures; (2) the appearance of a contracture at the knee joints; (3) the failure of injured muscles to regenerate and their replacement by hyperplastic connective tissue; (4) the development of subperiosteal thickenings of the diaphyses of certain bones and other anatomical changes. These last are treated in the second and third papers of this series.

EXPERIMENTS, MATERIAL, METHODS

The animals used were guinea-pigs. The weights of the vast majority, at the beginning of the experiment, were between 200 and 400 g. The basal diet was in the earlier experiments 720 g. oatmeal, 180 g. bran, 20 g. salt mixture, 100 g. dried yeast; later, the bran and yeast were changed to 160 and 80 g. respectively. To the basal diet we added ascorbic acid and radiostoleum. Carotene or carrots were given, as supplements of vitamin A, to the diets of those deprived of vitamin D. The supplements given in the different experiments are summarized in Table 1. In the diets which were partially deficient in vitamin C, our aim was to reduce the intake of the vitamin to as low a level as we could, while preventing obvious scurvy and death; in some experiments we gave 0·5 mg., in others 1 mg. ascorbic acid daily. The animals were weighed on alternate days, or sometimes daily.

The fractures were inflicted under ether anaesthesia with forceps resembling those used by Hertz (1936). Following Hertz, the fibula was chosen for fracturing because the tibia made an effective splint and there was usually good apposition of the ends. No open operation was required, but the method undoubtedly involved injury to the soft tissues by squeezing.

* Parts II and III will appear in subsequent issues of the *Journal*.

† This work was done while the first author was at the Department of Biology, St Bartholomew's Hospital Medical College, London.

In all experiments, except Exp. 2, a radiograph was taken to confirm that a fracture had been made, and further radiographs were taken later at intervals of from a fortnight to a month.

The animals were killed with chloroform. The operated limb, and often both posterior limbs and other parts, were fixed in Heidenhain's 'Susa' solution. Specimens were at first embedded by Apathy's celloidin-paraffin method, but later in paraffin; the latter method provided more complete series of sections. Sections were stained with haematoxylin and eosin, Heidenhain's 'Azan', Wilder's silver method, van Gieson, Giemsa, and other methods. When animals died before being killed, similar material was collected and preserved in either 10% formol or Susa.

Table 1 summarizes the nine experiments, showing how the diets were varied, the numbers of animals, the dates of the operation when one was performed, and the dates of death. 'Experiment 1' is really a list of all the dietetically normal animals, including some of those also listed as group 1 in Exps. 2–6, in which a fractured fibula was studied histologically.

PARTIAL DEFICIENCY OF VITAMIN C AND DEPRIVATION OF VITAMIN D, AND THE REPAIR OF FRACTURES

The diets deficient in vitamin D, in which the balance of salts was not altered, produced neither rickets nor any delay in callus formation at the fractures, nor indeed any other effect, whether alone or combined with a partial deficiency of vitamin C.

The effects of partial and complete vitamin C deficiencies on early stages in the repair of bone have been studied by Bourne (1942, 1943, 1944) and others (Hanke, 1935; Hertz, 1936; Israel, 1926; Israel & Fränkel, 1926; Jeney & Korpássy, 1934; Lexer, 1939; Roegholt, 1931–2; Schilowzew, 1928; Watanabe, 1924; Wolbach & Howe, 1925, 1926), but the influence of prolonged partial deficiencies, which we studied in Exps. 2–6 is less well known.

The radiographic findings and the results of the microscopical investigation will be discussed separately.

Radiographs

In Exp. 5, radiographs were taken of the operated limbs immediately after the operation and at 17, 32, 59, and 94 days later, and in Exp. 6 immediately after the fracture and at 19, 50 and about 80 days after the fracture. In group 1 (fully supplemented diets) of both experiments, the radiographs (Pl. 1, figs. 1, 3) taken at 17 or 19 days showed a callus with a narrow uncalcified line across it, while in those taken at 32 or 50 days no such line was visible and the callus was so reduced as to make the exact position of the fracture difficult to recognize. In groups 3 (partially deficient in vitamin C) and 4 (partially deficient in vitamin C and deprived of vitamin D), the amount of callus formed (at least at the site of the fracture) at 17 or 19 days was much less (Pl. 1, figs. 2, 4). In addition, the resorption of bone from the broken ends was usually greater than in the control animals (group 1) as shown by the greater width of the clear line between them. Even at 32 or 50 days, traces of the gap, or even a clear area right across the bone, were often visible (Pl. 1, fig. 2).

TABLE 1. *A summary of the dietary vitamin supplements given, of the times at which supplements were changed, of the times of operation, and of dates of death. Exp.* 1 *includes all animals, on fully supplemented diets throughout, whose fractured fibulae were sectioned*

Exp.	Group	No. of animals	Days of experiment	Vitamin C supplement	Vitamin D supplement	Vitamin A supplement	Day of operation	Days of experiment on which animals died or were killed (in brackets: number of animals)
1	—	34	—	Fully supplemented			1	1–7 (1 each), 9 (2), 10–14 (1 each), 15 (4) 16 (5), 63 (1), 81 (2), 83 (2), 84 (2), 85 (1), 94 (2), 101 (1)
2	1	10	1–14	Cabbage	6 drops radiostoleum weekly		1	25 (1), 30 (1), 33 (1), 42 (1), 53 (1), 60 (1), 65 (1), 85 (3)
			15–25	None	,,	,,		
			26–end	0·5 mg. A.A. daily*	,,	,,		
	2	4	1–end	Cabbage	6 drops radiostoleum weekly		1	85 (4)
3	1	7	1–end	10 mg. A.A. daily	6 drops radiostoleum weekly		1	16 (3), 102 (4)
	2	8	1–end	10 mg. A.A. daily	None	8 g. carrots weekly	1	16 (3), 18 (1), 54 (1), 102 (3)
	3	4	1–15	10 mg. A.A. daily	6 drops radiostoleum weekly		1	42 (1), 102 (3)
			16–32	0·5 mg. A.A. daily	,,	,,		
			33–60	1·0 mg. A.A. daily	,,	,,		
			61–end	0·5 mg. A.A. daily	,,	,,		
	4	12	1–15	10 mg. A.A. daily	None	8 g. carrots weekly	1	31 (1), 32 (1), 42 (1), 69 (1), 88 (1), 102 (2), 103 (5)
			16–32	0·5 mg. A.A. daily	,,	,, ,,		
			33–60	1·0 mg. A.A. daily	,,	,, ,,		
			61–end	0·5 mg. A.A. daily	,,	,, ,,		
4	2	4	1–end	10 mg. A.A. daily	None	8 g. carrots weekly	1	15 (1), 81 (3)
	4	4	1–14	10 mg. A.A. daily	,,	,,	1	81 (4)
			15–37	1·0 mg. A.A. daily	,,	,,		
			38–end	0·5 mg. A.A. daily	,,	,,		
5	1	4	1–end	10 mg. A.A. daily	6 drops radiostoleum weekly		27	121 (4)
	2	4	1–end	10 mg. A.A. daily	None	10 drops carotene twice weekly	27	52 (1), 91 (1), 107 (1), 121 (1)
	3	5	1–7	1·0 mg. A.A. daily	6 drops radiostoleum weekly		27	63 (1), 67 (1), 113 (2), 121 (1)
			8–18	None	,,	,,		
			19–end	0·5 mg. A.A. daily	,,	,,		
	4	5	1– 7	1 mg. A.A. daily	None	10 drops carotene twice weekly	27	45 (1), 47 (1), 63 (1), 93 (1), 120 (1)
			8–18	None	,,	,, ,,		
			19–end	0·5 mg. A.A. daily	,,	,, ,,		

Exp.	Group	No. of animals	Days of experiment	Vitamin C supplement	Vitamin D supplement	Vitamin A supplement	Day of operation	Days of experiment on which animals died or were killed (in brackets: numbers of animals)
	5	2	1– 7	1 mg. A.A. daily		6 drops radiostoleum weekly	None	62 (1), 121 (1)
			8–18	None		,, ,,		
			19–end	0·5 mg. A.A. daily		,, ,,		
	6	2	1– 7	1·0 mg. A.A. daily	None	10 drops carotene twice weekly	None	61 (1), 63 (1)
			8–18	None	,,	,, ,,		
			19–end	0·5 mg. A.A. daily	,,	,, ,,		
6	1	4	1–end	10 mg. A.A. daily		6 drops radiostoleum weekly	24	98 (1), 105 (2), 109 (1)
	2	4	1–end	10 mg. A.A. daily	None	10 drops carotene twice weekly	24	47 (1), 79 (1), 85 (1), 99 (1)
	3	4	1– 7	1·0 mg. A.A. daily		6 drops radiostoleum weekly	24	49 (1), 51 (1), 95 (1), 109 (1)
			8–16	None		,, ,,		
			17–end	0·5 mg. A.A. daily		,, ,,		
	4	5	1– 7	1·0 mg. A.A. daily	None	10 drops carotene twice weekly	24	30 (2), 51 (1), 65 (1), 85 (1)
			8–16	None	,,	,, ,,		
			17–end	0·5 mg. A.A. daily	,,	,, ,,		
	5	2	1–7	1·0 mg. A.A. daily		6 drops radiostoleum weekly	None	95 (1), 109 (1)
			8–16	None		,, ,,		
			17–end	0·5 mg. A.A. daily		,, ,,		
	6	2	1– 7	1·0 mg. A.A. daily	None	10 drops carotene twice weekly	None	72 (1), 109 (1)
			8–16	None	,,	,, ,,		
			17–end	0·5 mg. A.A. daily	,,	,, ,,		
7	1	12	1–10	None		6 drops radiostoleum weekly	None	54 (1), 62 (1), 64 (2), 66 (8)
			11–end	0·5 mg. A.A. daily		,, ,,		
	2	8	1–10	None		6 drops radiostoleum weekly	None	72 (1), 86 (1), 107 (6)
			11–66	0·5 mg. A.A. daily		,, ,,		
			67–end	10 mg. A.A. + cabbage daily		,, ,,		
8	1	6	1–end	None		6 drops radiostoleum weekly	None	16 (1), 17 (5)
	2	8	1–16	None		6 drops radiostoleum weekly	None	66 (8)
			17–end	15 g. cabbage daily		,, ,,	None	
9	—	16	1– 8	1·0 mg. A.A. daily		6 drops radiostoleum weekly	27	29 (3), 31 (3), 34 (3), 37 (2), 40 (2), 42 (1), 44 (2)
			9–18	None		,, ,,		
			19–end	0·5 mg. A.A. daily		,, ,,		

* A.A. = Ascorbic acid

Microscopical investigation

Sections confirmed these differences between the dietetically normal and partially vitamin C-deficient animals.

Normal animals

It is not necessary to give a full description of normal fracture repair, but certain points must be mentioned for comparison with events in the animals on experimental diets. The formation of new bone did not begin at the fracture itself, but at some distance from it in the swollen cambial layer of the periosteum; later, it gradually approached the fracture site. At about the end of the first week after the operation, cartilage might begin to appear in the densely cellular tissue in which the broken ends were now embedded. One of the two animals (Pl. 2, fig. 5) killed at 9 days showed a considerable quantity of cartilage forming in the fracture region, while the subperiosteal bone had reached the fracture and trabeculae growing out from the proximal and distal fragments, each towards the other, tended to link up around it. In the other 9-day specimen (Pl. 2, fig. 6) killed at this time, neither bone nor cartilage had yet appeared at the fracture site, though subperiosteal bone was actively developing at a short distance from the broken ends. In all the animals killed at from 10 to 14 days (Pl. 2, fig. 7), bone formation had spread to the fracture, and trabeculae had formed across it, making a sort of basket of trabeculae connecting the proximal and distal subperiosteal tissues. At first no bone formed centrally, between the broken ends themselves, but centrally situated trabeculae were present after 14 days. Cartilage was present in most cases. It tended to form in the peripheral parts of the cellular tissue which joined the fragments before the appearance of bone, but it was later often found in the central region between the ends. It was finally eroded and replaced by bone. In succeeding days (Pl. 2, fig. 8) the trabeculae of the callus became stronger and more numerous, and the cartilage, except for vestiges enclosed in bony trabeculae, disappeared.

The condition of the fractured region was investigated in eleven animals killed at times ranging from 81 to 101 days after the operation (Pl. 3, fig. 9). In all these the callus was recognizable in longitudinal sections from its disorderly structure. It differed from the callus of 2–3 weeks after fracture in two important respects. First, further deposition of bone on the trabeculae, and doubtless the formation of new trabeculae, had transformed the lightly built early callus into massive bone in which the solid substance predominated over the vessel-containing spaces, the opposite of the earlier condition. Secondly, the marked local expansion of the fibula, made by the early callus, had disappeared, and sections showed that the projecting part had been removed by osteoclastic resorption from the surface.

Partially vitamin C-deficient animals

Fracture repair in animals placed on the experimental diets before the infliction of the fracture was studied in Exps. 5, 6 and 9. The material consisted of longitudinal sections through the fractured region in animals killed at the following number of days after operation (the numbers in brackets give the numbers of days between

beginning the experimental diets and death): 14 (40), 16 (42), 18 (45), 20 (47), 27 (51), 36 (63, two animals), 40 (67), 61 (85), 86 (113), 93 (120).

The tendency, seen in the radiographs, of many animals on the experimental diets to show more resorption at the broken ends than the controls did, was confirmed by the animal killed at 14 days (Pl. 3, fig. 10) in which there was a long gap between the fragments, filled by a cellular and fibrous tissue. No repair had yet begun at the fracture itself. In none of the animals on the normal diet and killed after 9 days was the repair process still in so early a stage; in all, cartilage had formed and bony trabeculae were crossing the gap.

In the experimental animals which died or were killed at 16, 18 (Pl. 3, fig. 11) and 20 days after the operation, a few trabeculae had joined across the gap, making a feeble connexion, but there was no cartilage, and the repair accomplished was very much less than in the normal animals. There was, however, great variation in the effect of the diet, and the animal which died at 18 days showed repair indistinguishable from that expected in an animal on a normal diet.

In most of the material from animals kept for longer periods, the new subperiosteal bone which, as in normal animals, first developed at some distance from the site of the fracture, had increased enormously and had formed a great thickening often extending along the whole length of the diaphysis. These diaphyseal thickenings, which will be described in detail in the second paper of this series, were trabecular structures resembling lightly built calluses of great extent. In the two animals at 36 days after the operation, and in that at 61 (Pl. 3, fig. 12), the two pieces of the fibula were connected by the new bone extending over them like an immense callus. In the one which died at 93 days a pad of cartilage remained, still unresorbed, between the ends (Pl. 3, fig. 13). In those at 40 and 86 days, in which no large thickening had developed, they were weakly joined by small calluses developed between and around their ends. The chief fact of interest is that, whether the region of the fracture was, or was not, enclosed in a large mass of new bone, the repair of the fracture was never completed as it was in the controls, for the callus, large or small, always remained of light trabecular construction and was never consolidated as it was in the animals on properly supplemented diets. Compare Pl. 3, figs. 12 and 13, with fig. 9.

Pl. 4, fig. 14, shows the fractured fibula of an animal of Exp. 4, group 4, in which the partially deficient diet did not begin until 2 weeks after the operation, and in which there was no period of total deficiency. Nevertheless, the callus, which must have been formed when the partial deficiency began, failed to consolidate. At least one other case behaved similarly.

STIFFNESS OF THE KNEES

The most constantly observed change at the joints was a development of stiffness at the knees. This occurred in the great majority of partially vitamin C-deficient animals. Thus, in Exp. 9, in which the condition was most completely recorded, some degree of stiffness was found in both legs of all sixteen animals. It was never seen in guinea-pigs adequately supplied with vitamin C, and we did not notice it in other joints. Usually the leg was fixed in flexion, but sometimes in extension; in

severe cases we were unable to move the joint without using force which we feared would cause a fracture, while in others the knee could be moved with less violence but with evident pain. The condition has been described by Meyer & McCormick (1928), who give a photograph. Stiffness did not occur in animals provided with vitamin C but deprived of vitamin D.

The stiffening of the knees seems to be one of the earliest signs of a partial deficiency of the vitamin. In Exps. 5, 6 and 9 it was already present on the day of the operation, that is on the 24th and 27th days of the experimental diet, which had included a period of total deficiency of vitamin C followed by partial deficiency. Unfortunately, we do not know whether, in the present experiments, the stiffness appeared during the period of total deficiency or not, but in more recent experiments by one of us (E. K.) it did not appear during total deprivation lasting 18 days. That total deficiency is not necessary for the appearance of stiffness is shown by Exps. 3 and 4, in which there was no total deprivation, and in which stiffness appeared in at least thirteen out of thirty-two legs examined.

Stiffness is also not a result of the operation, for in Exps. 3–6, among thirty legs operated, stiffness developed in nineteen but not in the other eleven, while of twenty-six unoperated legs, seventeen showed stiffness.

There was a distinct correlation between the right and left sides of the same animal: in the experiments just mentioned, fifteen showed stiffness of both legs, eight of neither, and only three showed it in one leg and not in the other. Stiffness thus depends rather on the diet than on the injury.

Meyer & McCormick (1928) attributed stiffness to changes which they observed in the spinal cord. We made no study of the nervous system, but found that the knees remained stiff after death, and in Exp. 9 we studied the effect of removing the muscle crossing the knee joint. This lessened the stiffness in ten cases but left it unaffected in nine. We are thus disinclined to regard the condition as wholly of central nervous origin. In the next section we shall describe a swelling of the soft tissues of the shank, with degeneration of the muscles and their replacement by a mass of hyperplastic connective tissue. This condition would certainly help to prevent active movement of the limbs and in extreme cases the connective tissue, by packing around the joint and thickening its capsule, may have lessened freedom of movement. The articular surfaces themselves nearly always appeared normal. An attempt to correlate stiffness of the knees with pathological changes in the musculature has an indefinite result. In the animals more severely affected by the partial deficiency, there were such characteristic scorbutic changes as haemorrhages about the knee and even into the joint cavity, and this doubtless contributed to the stiffness.

We incline to the view that stiffness is caused locally rather than in the central nervous system, and that a number of histological changes contribute, these including degenerative and oedematous changes in the muscles, and the formation of hyperplastic connective tissue at and around the joint.

MUSCLE DEGENERATION AND CONNECTIVE TISSUE HYPERPLASIA

When guinea-pigs in which a fibula had been fractured and which had been kept for long periods (up to 4 months) on diets deficient in vitamin C were killed, it was frequently noticed that one or both posterior limbs were swollen in the shank region, with tightly drawn skin, a change which did not occur in dietetically normal controls. On dissection, the flesh had a curious gelatinous feel and it was difficult to separate the muscles from one another; often they seemed to have been replaced by a tissue of a different character.

Structure of the altered tissue

The altered tissue was studied in Exps. 3–6. Transverse sections of the swollen shank regions of animals from the partially vitamin C-deficient groups showed that there had been a great increase in the amount of loose connective tissue which, in severe cases, had largely replaced the muscles (Pl. 4, figs. 15, 16). In animals kept for such long periods as 3 months after the operation this tissue had the following character. The cells present were almost all of one kind, and resembled fibroblasts. The intercellular tissue components were a fibrillar framework and the tissue fluid. The fibrillar framework varied a good deal in character. The fibrils were extremely fine, and were often so knitted or matted together as to form the membranous walls of a honeycomb system, in the multitudinous, and of course communicating, cavities of which was the tissue fluid (Pl. 5, fig. 23). The picture was reminiscent of an argyrophil reticulum rather than of a mature areolar connective tissue with its interlacing collagen fibres as its dominant feature. The fibrils stained black with Wilder's silver method, except for the very finest, which we did not succeed in staining at all but made visible by mounting in 'Euparal'. The fibrils took only a weak blue colour with Azan and did not stain with van Gieson. As well as this architecture of extremely fine fibrils, collagen fibres were often also present, and might be a prominent feature in the histological picture.

Besides the formation of the hyperplastic connective tissue replacing the muscles, the normal connective tissue structures, such as fascia and the fibrous layers of periosteum, tended to break down. Fasciae might become completely unrecognizable. The fibrous layers of periosteum never broke down completely, but areas could be found in which the limiting fibrous membrane between the osteogenic tissue and the surrounding hyperplastic connective tissue had suffered dissolution, leaving no histologically recognizable barrier between them (Pl. 4, fig. 17). A similar fate might befall the interosseous ligament between the tibia and fibula, and even tendons.

When muscles were present in the section, depending in part on whether or not they were removed at dissection, they might be normal. On the other hand, they were often oedematous, the fibres not being packed closely together as in a normal muscle, but having wide spaces between them. The connective tissue framework of the muscle might be increased in quantity, as though the muscle were being invaded by the extra-muscular connective tissue, or its own connective tissue proliferating. The muscle fibres had often shrunk away from their envelopes and had lost their characteristic internal structure, the myofibrils being fused into a homogeneous mass.

In places, groups of more or less isolated muscle fibres might be seen wandering

through the hyperplastic connective tissue by which they were surrounded and separated from one another. Such muscle fibres usually seemed to have retained their structure, showing nuclei and striations, but were very small, often extremely so. We find it very difficult to decide whether they were fibres in process of destruction by atrophy, or regenerating fibres which had failed to grow to anything like their normal final size.

Vascularization of the hyperplastic connective tissue was always poor; vessels were few and often without blood corpuscles, especially the arteries. Arteries were frequently seen to have their lumina partially or completely blocked by thickening and vacuolation of the endothelium (see below). Often, but by no means always, large numbers of red blood corpuscles could be seen lying in the tissue spaces of large areas of the connective tissue.

Development of hyperplastic connective tissue

The origin and development of this tissue was studied in Exp. 9, in which sixteen young guinea-pigs on a partially vitamin C-deficient diet were killed after fracture of the fibula at intervals given in Table 1. Transverse serial sections were cut across the operated limbs, above and below the site of fracture, and a few longitudinal series were also made.

There was little or no hyperplasia of the connective tissue in animals killed at 3 days after the fracture, but its formation had begun at 5 days, was more obvious at 8 days, and was large in the animals killed at 11 days and later.

The formation of the new tissue was closely bound up with degenerative changes in the muscle. These were seen in the animals killed at 3, 5 and 8 days after the operation, and in one killed at 11 days. In animals killed later, muscular degeneration was much less apparent, but much muscle had disappeared. These changes resulted in the total destruction, so far as could be seen in transverse sections, of many or all of the muscle fibres in entire muscles. The earliest recognizable departure from the normal was an oedema of the muscles in which the fascicles, and also individual muscle fibres, became widely separated from one another (Pl. 5, fig. 18). So far, the individual muscle fibres appeared normal. This simple oedema was seen especially in the earlier killed animals, but was also to be seen much later; it could be, but was not necessarily, a prelude to the destructive changes next to be described. These began with a loss of nuclei and a disappearance of the fibrillar structure of the muscle fibres (Pl. 5, fig. 19). It was difficult to determine exactly what happened to the nuclei; they were either present and apparently normal, or absent. They did not become pycnotic, for such a change would have been easily recognizable, and we think some of them disappeared by a progressive loss of their staining power. In less injured muscle fibres, the nuclei survived and might later take part in what little regeneration occurred. The loss of fibrillar structure appeared as a fusion of the fibrils, making the fibres appear homogeneous in cross-section. This change was often accompanied by an increase in the diameter of the fibre and by a development of vacuoles; these appeared first as many tiny droplets, giving the cross-section of the fibre a pitted appearance (Pl. 5, fig. 20). Later, the droplets might coalesce and form a large central cavity by which the fibre might be blown out to a very large size. Vacuolation of the fibres was

common but not universal; a great many fibres acquired a 'fluffy' appearance and broke up into irregular fragments. Even in muscles in which most of the fibres were undergoing these changes, a few fibres here and there often seemed to remain normal.

While muscle fibres were degenerating, the muscles were invaded by cells from without. Most of these were rather large cells with much cytoplasm and oval or indented nuclei, of the type of mononuclear wandering cells. Mitotic figures were common among them. They were found in large compact masses, having a pseudo-epithelial appearance, in the peripheral parts of the sections. In life, these groups must have been close beneath the skin. From the masses, it was easy to trace migration of the cells to the muscles, into which they penetrated. Within the muscles, they applied themselves to the surfaces of the degenerating muscle fibres, and gave every appearance of being engaged in their destruction (Pl. 5, fig. 21). Multinucleate giant cells were also present, and similarly appeared to be engaged in attacking the degenerate fibres. At a somewhat later stage the muscle fibres, or many of them, had disappeared, while their endomysial or sarcolemmal envelopes persisted as empty tubes whose walls no longer gave with Azan the blue reaction so brilliantly seen in neighbouring normal muscles. The wandering cells could often be seen in occupation of these tubes in place of the original owners (Pl. 5, fig. 22).

While the destruction of muscle fibres was in progress, there was an increase in the fibroblasts between them. This was brought about partly by multiplication of cells in the original connective tissue of the muscle and partly, we suppose, by transformation of the wandering cells. Up to 8 days after the operation, degenerating muscles infiltrated by wandering cells are a predominant feature in the histological picture, while increase in the connective tissue is not yet striking, but from 11 days onwards such gross degenerative changes are scarce or absent while the striking histological feature is the great masses of young connective tissue cells occupying areas which had evidently been muscular, and in which traces of the envelopes of muscle fibres could often easily be seen. In such regions, the fibroblasts of the new connective tissue seem at first comparatively scarce, but there are mitotic figures among them. They multiply, and as they do so a delicate fibrillar reticulum forms, replacing the old architecture of the destroyed muscle, and this soon becomes unrecognizable.

At the same time, fibroblasts of the intermuscular connective tissues also proliferate and so contribute to the developing mass of new connective tissue.

A second mode of formation of the connective tissue was by proliferation of the fibroblasts between the muscle fibres of oedematous muscles whose fibres did not degenerate as described above but, we believe, were reduced in size and number by an atrophy which ended in the disappearance of many fibres.

Still a third mode of origin of the new tissue was by the organization of exudates, which were commonly found in the intermuscular connective tissue. By the penetration of fibroblasts into such exudates, they were built up into parts of the general connective tissue.

It was impossible to tell how much of the hyperplastic connective tissue formed by each of these modes of origin, because the final result was in each case the same, but the largest contribution was certainly made by the destruction of muscle fibres and their replacement by connective tissue.

The connective tissue, formed as just described, did not, within the limits of the

experiment (extending up to 18 days after the fracture), become identical in structure with the fully formed hyperplastic connective tissue which we have described in material fixed some 3 months after the operation. Large parts of it are much more densely cellular than it later becomes, and the honeycomb-like architecture, seen in the older material, has not yet developed, the fibrillar framework making a much more open net-work.

Regeneration of muscle fibres

In the developing hyperplastic connective tissue one could very often see a number of very small muscle fibres, wandering in an apparently undirected manner. Many of these were undoubtedly regenerating muscle fibres, for they showed multinucleate regeneration buds, but we suspect that some were fibres in course of atrophy. The regenerates, even in animals which lived for 3 months or more after the operation, remained very much smaller than normal fibres and were few in number; nothing suggested that any more complete regeneration of muscle fibres occurred, such as was described by Le Gros Clark & Blomfield (1945), and as we found in our dietetically normal animals. If such extensive regeneration had occurred in the dietetically partially deficient animals, the observations described above, of the replacement of large areas of muscle by connective tissue, could not have been made.

Comparison with dietetically normal animals

The changes described above in animals on diets partially deficient in vitamin C were also seen in those on fully supplemented diets (Exp. 1, and group 1 of Exps. 3–6), but there was a great quantitative difference.

In the three dietetically normal animals killed on the 1st, 2nd and 3rd days after the fracture, a considerable number of changed muscle fibres were found. These fibres showed loss of nuclei, loss of striations, and loss of myofibrils, giving the fibres a homogeneous appearance. Some fibres suffered fragmentation. The muscles were oedematous and there was an extensive polymorph infiltration. Whether there was markedly less of these changes in the first 3 days than was to be seen in the two partially deficient animals killed at 3 days, it is difficult to say.

In the animal killed at 4 days after the fracture, the polymorph infiltration was much less, there was more than the normal amount of connective tissue and this was very cellular and obviously recently formed. Since regenerating muscle fibres were present in it, it had evidently formed in place of destroyed muscle tissue.

In the legs of the remaining animals killed during the first 16 days after the fracture, muscle fibres undergoing degeneration were rare, but regenerating fibres were very numerous, growing through areas of young connective tissue.

We have little doubt that the amount of muscle tissue which was destroyed after suffering injury at the operation was much less in the dietetically normal animals than in the partially deficient, but no quantitative estimate was possible. It is certainly true that the amount of hyperplastic connective tissue formed in the limbs of the partially deficient animals was very much greater than in those on the normal diet; this difference must evidently reflect either a greater quantity of muscle destroyed in the partially deficient animals, or a greater quantity regenerated in the normals, or most probably both.

In dietetically normal animals killed 3 months or more after the operation, there was little or no connective tissue beyond the normal, and no regeneration in progress; the injured fibres had, apparently, long since been restored to the normal condition. This was, of course, in sharp contrast with the partially deficient animals in which the hyperplastic connective tissue was, at corresponding times, at its fullest development, in which there had been very much less regeneration of muscle fibres, and in which such regenerates as did occur failed to attain anything like the normal size.

Aetiology

It is natural to attribute the oedema, the exudates, and the muscular degenerations, to the injury inflicted by the fracture forceps. This is supported by the following figures.

In Exp. 9, in which we studied the development of the connective tissue hyperplasia, killing sixteen animals at various times after the operation, if the formation of the hyperplasia were a consequence of the operation, it would not be expected to occur until several days after it. This was the case, for it had not appeared in animals killed at 3 days, was beginning in those killed at 5 and 8 days, but was large at and after 11 days.

Again, if the hyperplasia were a result of the operation, it should have occurred in the operated and not in the unoperated limbs. Of the sixteen animals in Exp. 9, thirteen showed hyperplasia in the operated leg, and the remaining three, which were those killed at 3 days after the operation, showed in the muscles of the operated legs the changes which precede the formation of hyperplastic connective tissue. Of the sixteen unoperated legs, thirteen showed no sign of hyperplasia, while some hyperplasia was present in three (19 %). In two of these it was much less than on the operated side. In Exps. 5 and 6, nineteen animals on partially deficient diets suffered the operation on one side. There was formation of hyperplastic connective tissue in at least seven of the nineteen operated legs (37 %). In the same experiments, eight other animals were similarly dieted but not operated (groups 5 and 6). Among the thirty-five legs, made up of two from each of these eight and of the nineteen unoperated legs of the animals mentioned above, connective tissue hyperplasia developed in at least eight (23 %). In Exps. 3 and 4, which included nineteen animals kept on diets partially deficient in vitamin C, some connective tissue hyperplasia occurred in at least eight out of twenty-six operated legs (31 %), and in at least one out of twelve legs which were not operated (8 %). In this experiment the operation was performed 2 weeks before the diet was made partially deficient in vitamin C, and during this period repair was proceeding on a fully supplemented diet; nevertheless, the partial deficiency, late though it began, had the effect just stated.

We conclude that (1) the connective tissue hyperplasia developed more readily in operated than in unoperated legs, but (2) it could develop in legs which had suffered no operative interference.

The hyperplasia never developed in the legs of animals receiving 10 mg. of ascorbic acid daily.

The evidence clearly points to an inability of the musculature of the partially vitamin C-deficient guinea-pigs to regenerate after injury, and probably indicates

a greater susceptibility to injury in these animals, than in those supplied with adequate quantities of the vitamin. Why, then, was there in some cases a connective tissue hyperplasia in unoperated limbs? The only answer we can make to this is to suggest that the muscles, which the vitamin deficiency has made more liable to injury, can be so affected by traumatic agents, which would have much less effect on normal limbs, as to bring about the changes which we described above. Possible occasions of such injury were the animals' kicking during the taking of radiographs, in handling during administration of ascorbic acid, etc.

We have examined the blood vessels in sections through the legs of partially vitamin C-deficient and dietetically normal animals. In the operated legs of partially deficient animals the arteries especially were affected. The changes could be recognized in the animals killed at 3 days, and were seen in full development in those killed at 5 days. At this time, and later, many of the vessels were empty of corpuscles, or nearly so, especially the arteries. Again, chiefly in the arteries, the endothelial cells were swollen by vacuoles developed in the basal parts of the cells, between the nuclei and the internal elastic membrane. The vacuoles cause the cells to project into the lumen of the vessel (Pl. 5, figs. 24, 25). Small arteries may be blocked by projecting, and even desquamated, endothelial cells. Delicate strands seen in the sections crossing the lumen from side to side were difficult to interpret with certainty; they seemed to be the walls of very greatly expanded endothelial vacuoles. Vacuoles might also appear beneath the internal elastic membrane and among the smooth muscle fibres. Occasionally, the vacuolation here became so intense that the muscle fibres were loosened up and separated from one another (Pl. 5, fig. 26); rarely, the disintegration process might go so far as a complete breakdown of the cross-section of the vessel (Pl. 5, fig. 27), which might then be histologically recognizable only by tracing a connexion, through serial sections, with its more normal parts. The veins usually appeared normal, and contained blood, with vacuolate and swollen endothelium. Lymphatics were numerous and often greatly expanded; mitotic figures were often seen in their walls.

Examination of the blood vessels in those legs of guinea-pigs which were killed or died at times ranging up to 3 or 4 months after the operation, and which showed hyperplasia of the connective tissue, also showed that the conditions just described in animals killed during the first 18 days after the operation had persisted. The hyperplastic connective tissue was always nearly avascular, and most of the blood vessels (especially the arteries) in or near it were empty or nearly so, with vacuolate and swollen endothelium. When, on the other hand, there was no development of hyperplastic connective tissue, the blood vessels were normal.

Comparison with the operated legs of dietetically normal animals, and especially with those killed in the first 3 weeks, showed that it would be almost true to say that the vascular changes just described did not occur in these animals. In some specimens, however, especially in those whose legs showed any large accumulation of connective tissue, empty vessels, especially arteries, with vacuolate endothelium, could be found. But this was far less frequent than in the partially deficient animals and, when present, was less severe.

The present experiments do not enable us to decide whether the partial deficiency of vitamin C, in preventing adequate regeneration of injured muscles and causing

their replacement by connective tissues, acted directly, by increasing the sensitivity of muscle fibres to injury and/or by reducing their power of recovery and regeneration, or indirectly, as by causing interference with the vascular supply. Le Gros Clark & Blomfield (1945) found that interruption of the arterial supply to the muscles of otherwise normal animals led to degeneration of the muscle fibres and their temporary replacement by connective tissue; when an adequate collateral blood supply had been established, regeneration re-established the muscle and the excess connective tissue melted away. This suggests that the primary effect, in our partially deficient animals, may have been on the blood vessels. The use of the fracture forceps in the operation inevitably injured the muscles by squeezing them, causing haemorrhages by rupturing capillaries and other vessels. Haemorrhagic areas are very common in the muscles of our partially deficient animals, but the degenerative changes we have described often occurred in their absence. The vascular stagnation resulting from the rupture of many small vessels, and the oedematous condition of the muscles, might be expected to cause such anoxaemia as was presumably responsible for the muscular degeneration in Le Gros Clark & Blomfield's experiments, and might also take the blame for the closely similar changes in our material. However this may be, the blood vessels, or at least the arteries, are apparently more liable to injury in the partially deficient animals than in the dietetically normal controls. Meyer, in scorbutic animals, mentions changes in blood vessels similar to those we have described, but unrelated to any known injury and to hyperplastic connective tissue.

Our guinea-pigs were not supplied with any special supplement of vitamin E, and a number of authors (Einarson & Ringsted, 1938; Evans & Burr, 1928; Evans, Emerson & Telford, 1938; Olcott, 1938; Pappenheimer, 1939, 1943) have described degenerative muscular changes, in various species, when deprived of this vitamin. In some respects the changes described above resemble these descriptions, and the question arises whether the changes in our vitamin C-partially deficient animals could have been due to lack rather of vitamin E than of vitamin C. A number of facts tell against this idea. First, the changes in our material were local, whereas those caused by vitamin E deficiency are widely spread through practically the whole skeletal musculature. Secondly, authors describing the muscles in vitamin E deficiency do not mention the wholesale local replacement of muscles by hyperplastic connective tissue. Thirdly, and most convincingly, ascorbic acid protected our animals against the changes we have described, and would not do so against those of vitamin E deficiency.

SUMMARY

1. In partial vitamin C deficiency the formation of callus at fractures of the fibula was slower, and the amount of callus formed was at first less than in dietetically normal animals.

2. Whereas in normal animals the callus later consolidated into compact bone by thickening of the trabeculae, in the partially deficient animals this did not occur; the callus might become extremely extensive, covering the whole diaphysis (see the second paper of this series), but always retained a lightly built, trabecular structure.

3. In a large proportion of animals kept on partially vitamin C-deficient diets, the knees became stiff and could be bent only painfully and with difficulty. Evidence is presented indicating that this condition was caused at least in part by local histological changes.

4. The manner of fracturing the fibula caused some injury to muscle, and damaged muscle fibres degenerated. In dietetically normal animals they quickly regenerated. In partially vitamin C-deficient animals, much more muscle degenerated than in dietetically normal animals, and the muscular tissue so lost was replaced by large masses of hyperplastic connective tissue and did not regenerate. A large part of the limb musculature might thus disappear. The process of degeneration of the muscle fibres, and the development of the hyperplastic connective tissue, are described.

5. The degeneration of muscles and their replacement by hyperplastic connective tissue occurred more readily in operated legs (in which the muscles were certainly damaged) than in unoperated, but it was also seen in unoperated limbs, though less frequently. It is suggested that the muscles of partially vitamin C-deficient animals are more liable to injury than those of normal animals, and can be damaged by traumatic agents which do not affect those of normals. Possible occasions of such injury were the animal's kicking when being anaesthetized before radiographs were taken.

6. The hyperplastic connective tissue was always avascular or nearly so. Examination of the blood vessels, during the period of muscle degeneration, showed abnormalities especially in the arteries, and these are described. The vessels were found to be in the same condition in the hyperplastic connective tissue of animals which lived for months after the operation. The evidence does not permit us to say whether the replacement of muscles by connective tissue reflected a direct effect of the partial deficiency of vitamin C on the muscle fibres and their ability to regenerate, or an indirect effect through the blood vessels; a certain resemblance of the histological picture to that described by Le Gros Clark & Blomfield, after experimental interruption of the arterial supply, suggests the latter.

REFERENCES

Bourne, G. (1942*a*). The effect of ascorbic acid (vitamin C), calcium ascorbate, and calcium gluconate on the regeneration of bone in rats. *Quart. J. exp. Physiol.* **31**, 319–331.

Bourne, G. (1942*b*). The effect of graded doses of vitamin C upon the regeneration of bone in guinea-pigs on a scorbutic diet. *J. Physiol.* **101**, 327–336.

Bourne, G. (1942*c*). Vitamin C and repair of injured tissue. *Lancet*, **243**, 661–664.

Bourne, G. (1943). Some experiments on the possible relationship between vitamin C and calcification. *J. Physiol.* **102**, 319–328.

Bourne, G. (1944). Relative importance of periosteum and endosteum in bone healing, and relationship of vitamin C to their activities. *Proc. R. Soc. Med.* **37**, 275–279.

Einarson, L. & Ringsted, A. (1938). *Effect of Chronic Vitamin E Deficiency on the Nervous System and the Skeletal Musculature in Adult Rats.* Pp. 1–153. Oxford University Press.

Evans, H. M. & Burr, G. O. (1928). Development of paralysis in the suckling young of mothers deprived of vitamin E. *J. biol. Chem.* **76**, 273–297.

Evans, H. M., Emerson, G. A. & Telford, I. R. (1938). Degeneration of cross striated musculature in vitamin E-low rats. *Proc. Soc. exp. Biol., N.Y.*, **38**, 625–627.

Hanke, H. (1935). Experimentelle Untersuchungen über die Beeinflussung der Knochenregeneration durch Vitamin C. *Dtsch. Z. Chir.* **245**, 530–545.

Hertz, J. (1936). *Studies on the Healing of Fractures with Special Reference to the Significance of the Vitamin Content of the Diet*, pp. 1–286. Oxford University Press.

ISRAEL, A. (1926). Avitaminose und Knochenbrüchheilung. *Arch. klin. Chir.* **142**, 145–154.
ISRAEL, A. & FRÄNKEL, R. (1926). Versuche über den Einfluss der Avitaminose auf die Heilung von Knochenbrüchen. *Klin.-Ther. Wschr.* **5**, 94–97.
JENEY, A. & KORPÁSSY, B. (1934). Verzögerte Heilung der Haut und Knochenwunden bei Skorbuttieren. Erfolgreiche Behandlung mit Ascorbinsäure. *Zbl. Chir.* **61**, 2836–2847.
KODICEK, E. & MURRAY, P. D. F. (1943). Influence of a prolonged partial deficiency of vitamin C on the recovery of guinea-pigs from injury to bones and muscles. *Nature, Lond.*, **151**, 395–396.
LE GROS CLARK, W. E., & BLOMFIELD, L. B. (1945). The efficiency of intramuscular anastomoses, with observations on the regeneration of devascularized muscle. *J. Anat., Lond.*, **79**, 15–32.
LEXER, E. W. (1939). Die Bedeutung des Vitamin C für die Callusbildung und reaktive Gefässfülle. *Arch. klin. Chir.* **195**, 611–625.
MEYER, A. W. & MCCORMICK, L. M. (1928). Studies on scurvy. *Stanford Univ. Publ.* **2**, no. 2, 1–107.
MURRAY, P. D. F. & KODICEK, E. (1946). Some histological effects of partial deficiency of vitamin C on healing processes: the influence on bone repair. *Proc. nutrit. Soc.* **4**, 200–203.
OLCOTT, H. S. (1938). The paralysis in the young of vitamin E-deficient female rats. *J. Nutrit.* **15**, 221–225
PAPPENHEIMER, A. M. (1939). The pathology of nutritional muscular dystrophy in young rats. *Amer. J. Path.* **15**, 179–183.
PAPPENHEIMER, A. M. (1943). Muscular disorders associated with deficiency of vitamin E. *Physiol Rev.* **23**, 37–50.
ROEGHOLT, M. N. (1931–2). Der Einfluss der A-, B-, C-, D-Vitamine und der Elemente Ca und P auf die Heilung von Knochenbrüchen. *Arch. klin. Chir.* **168**, 783–805.
SCHILOWZEW, S. P. (1928). Der Einfluss der Avitaminose auf die Heilung von Knochenbrüchen. *Dtsch. Z. Chir.* **209**, 320–325.
WATANABE, T. (1924). Experimentelle Untersuchungen über den Einfluss der Avitaminose auf die Heilung von Knochenfrakturen. *Virchows Arch.* **251**, 281–296.
WOLBACH, S. & HOWE, P. R. (1925). The effect of the scorbutic state upon the production and maintenance of intercellular substances. *Proc. Soc. exp. Biol., N.Y.*, **22**, 400–402.
WOLBACH, S. & HOWE, P. R. (1926). Intercellular substances in experimental scorbutus. *Arch. Path. Lab. Med.* **1**, 1–24.

EXPLANATION OF PLATES

List of abbreviations used in plates: *cal.* callus; *cart.* cartilage; *fib.* fibular; *fract.* position of fracture; *n.b.* new bone; *peri.* periosteum; *tib.* tibia.

PLATE 1

Fig. 1. Normal animal. Stages in the repair of the fractured fibula; from Exp. 6, group 1.
Fig. 2. Partially vitamin C-deficient animal. Stages in the repair of the fractured fibula; from Exp. 6, group 4.
Fig. 3. Normal animal. Stages in the repair of the fractured fibula; from Exp. 5, group 1.
Fig. 4. Partially vitamin C-deficient animal. Stages in the repair of the fractured fibula; from Exp. 5, group 3.

PLATE 2

Fig. 5. Normal animal. Fibula, 9th day after fracture, showing cartilage (lower right) in the developing callus. Giemsa. ×35. From Exp. 1.
Fig. 6. Normal animal. Fibula, 9th day after fracture. No callus at fracture, but new bone on fibula at some distance from it, and on tibia. Tibia and its marrow on left. Haematoxylin and eosin. ×17. From Exp. 1.
Fig. 7. Normal animal. Fibula, 14th day after fracture, showing callus. Azan. ×38. From Exp. 1.
Fig. 8. Normal animal. Fibula, 16th day after fracture. Haematoxylin and eosin. ×25. From Exp. 1.

PLATE 3

Fig. 9. Normal animal. Fibula, 101st day after fracture. Bracket indicates consolidated callus. Haematoxylin and eosin. ×24. From Exp. 6, group 1.
Fig. 10. Partially vitamin C-deficient animal. Fibula, 14th day after operation, 40th day of experiment. Extensive resorption and no callus at site of fracture; new subperiosteal bone forming on both stumps. Azan. ×27. From Exp. 9.
Fig. 11. Partially vitamin C-deficient animal. Fibula, 18th day after fracture and 45th day of experiment. Azan. ×58. From Exp. 5, group 4.

Fig. 12. Partially vitamin C-deficient animal. Fibula and part of tibia, 61st day after fracture and 85th day of experiment. Haematoxylin and eosin. ×12. From Exp. 6, group 4.

Fig. 13. Partially vitamin C-deficient animal. Fibula and part of tibia, 93rd day after operation, 120th day of experiment. Haematoxylin and eosin. ×15. From Exp. 5, group 4.

PLATE 4

Fig. 14. Partially vitamin C-deficient animal. Fibula and part of tibia (right), 81st day after operation, 66th day of experimental diets. Haematoxylin and eosin. ×20. From Exp. 4, group 4.

Fig. 15. Partially vitamin C-deficient animal. Part of transverse section of shank, showing hyperplastic connective tissue, 102nd day after operation and 87th day of experimental diets. Haematoxylin and eosin. ×17. From Exp. 3, group 3.

Fig. 16. From the same specimen as fig. 15, showing hyperplastic connective tissue. Azan. ×60.

Fig. 17. Partially vitamin C-deficient animal, 81st day after operation, 67th day after beginning experimental diets. An area of tibial surface where the periosteum has dissolved in the surrounding hyperplastic connective tissue. Haematoxylin and eosin. ×175. From Exp. 4, group 4.

PLATE 5

Fig. 18. Partially vitamin C-deficient animal. An oedematous muscle, and, below, the fibular periosteum, 4th day after operation and 29th day of experiment. Haematoxylin and eosin. ×135. From Exp. 9.

Fig. 19. Partially vitamin C-deficient animal. Early degeneration of muscle fibres which show neither nuclei nor myofibrils, 4th day after operation, 29th day of experiment. Haematoxylin and eosin. ×580. From Exp. 9.

Fig. 20. Partially vitamin-C deficient animal. Degenerating muscle fibres showing 'pitting' by small vacuoles, and fragmentation of fibres, 6th day after operation, 31st day of experiment. Azan. ×600. From Exp. 9.

Fig. 21. Partially vitamin C-deficient animal. Degenerating muscle fibres under attack by mono- and multi-nucleate cells, 6th day after operation and 31st day of experiment. Azan. ×440. From Exp. 9.

Fig. 22. Partially vitamin C-deficient animal. Empty envelopes of muscle fibres occupied by wandering cells, 6th day after operation, 31st day of experiment. Haematoxylin and eosin. ×575. From Exp. 9.

Fig. 23. Partially vitamin C-deficient animal. The hyperplastic connective tissue, from the same specimen as fig. 15. Azan. ×745. From Exp. 3, group 3.

Fig. 24. Partially vitamin C-deficient animal. An artery with vacuolation of the endothelium, 12th day after operation, 37th day of experiment. Haematoxylin and eosin. ×210. From Exp. 9.

Fig. 25. Partially vitamin C-deficient animal. An artery with vacuolate and thickened endothelium, 4th day after operation, 29th day of experiment. Haematoxylin and eosin. ×210. From Exp. 9.

Fig. 26. Partially vitamin C-deficient animal. An artery showing vacuolation in both endothelium and connective tissue of the wall, 19th day after operation, 44th day of experiment. Haematoxylin and eosin. ×210. From Exp. 9.

Fig. 27. Partially vitamin C-deficient animal. Remains of a degenerate artery, only recognizable by tracing through serial sections, 15th day after operation, 40th day of experiment. Haematoxylin and eosin. ×210. From Exp. 9.

Fig. 1.

Fig. 2.

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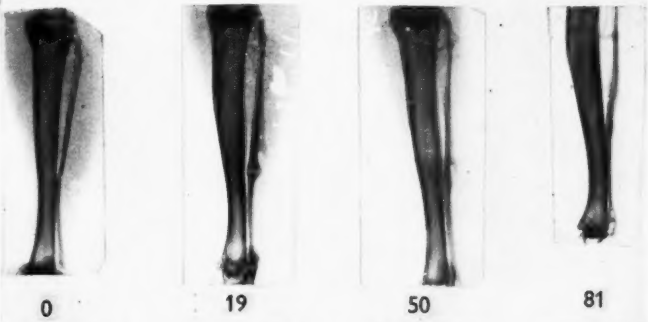

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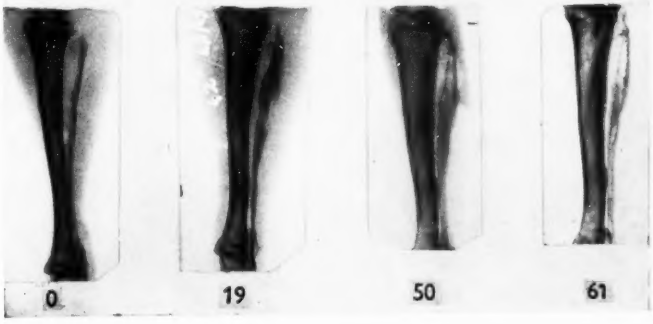
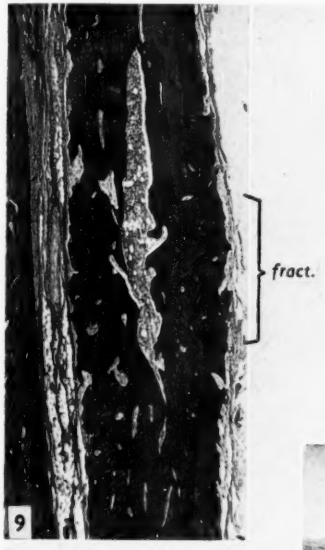


MURRAY AND KODICEK—BONES, MUSCLES AND VITAMIN C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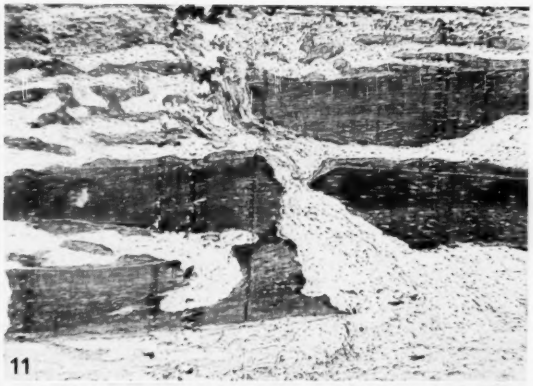
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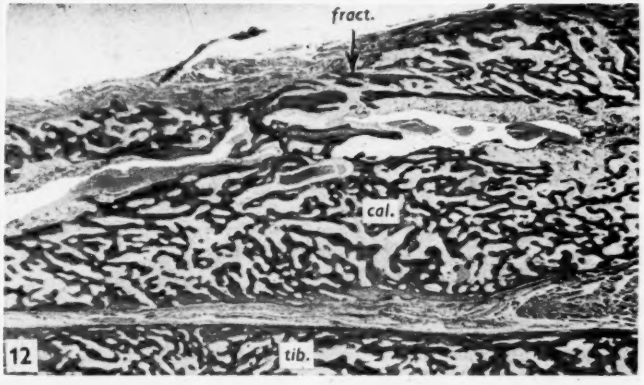
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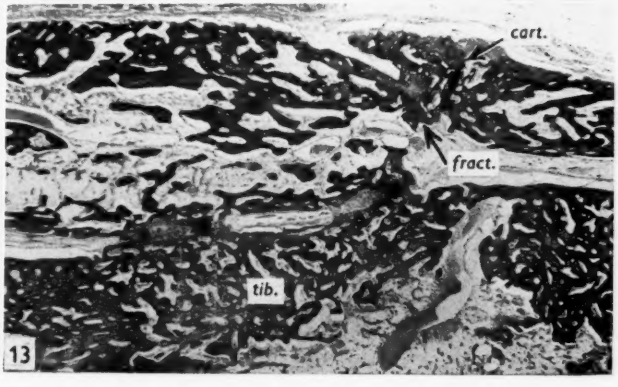
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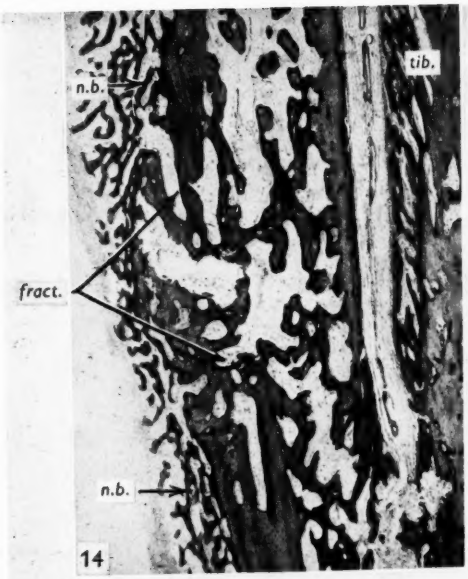
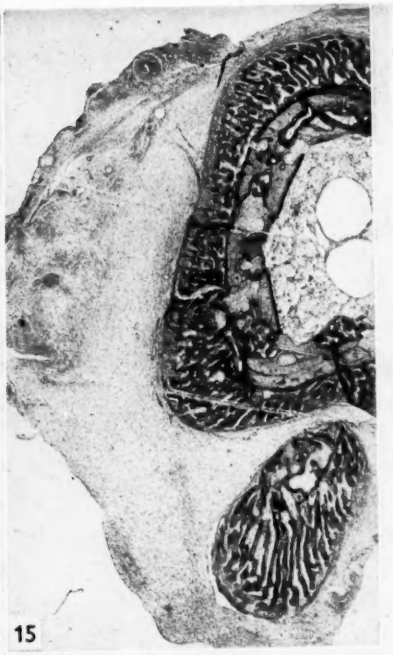
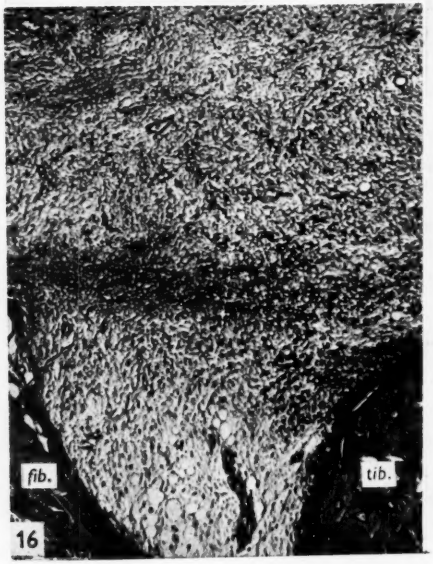
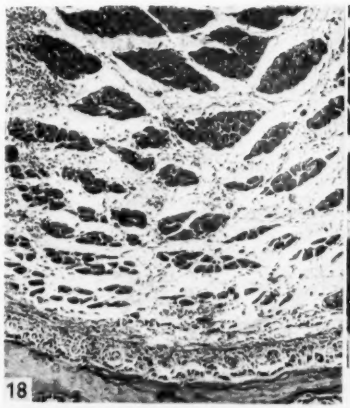






MURRAY AND KODICEK—BONES, MUSCLES AND VITAMIN C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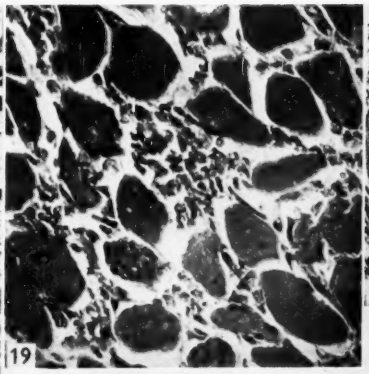
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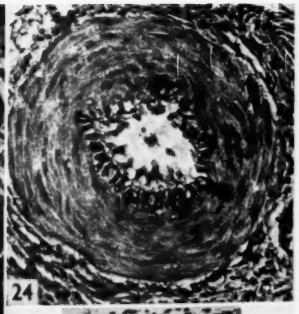
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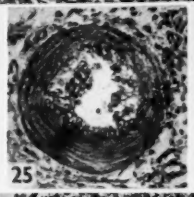
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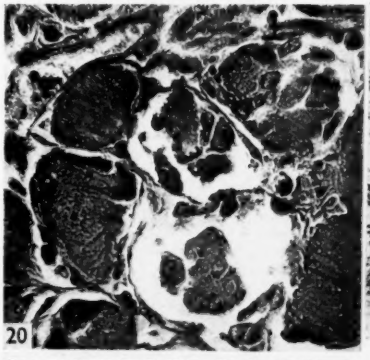
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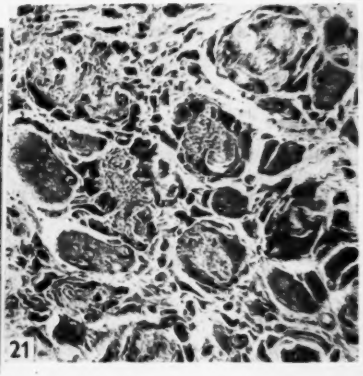
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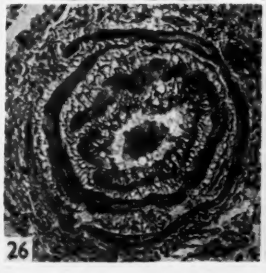
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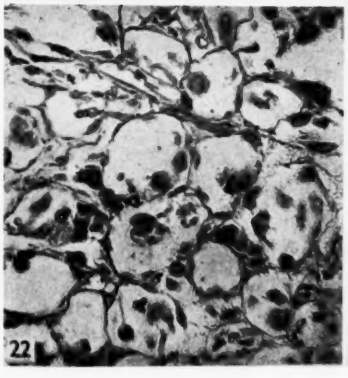
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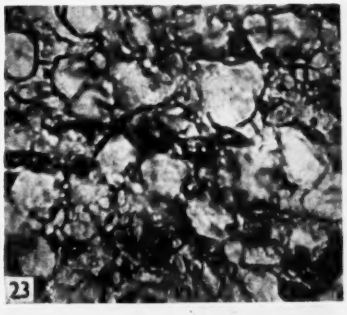
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ANATOMICAL NOTE

THE LOWER LIMIT OF THE SPINAL CORD IN SOUTH INDIAN FOETUSES

By V. SITARAMA RAO, *Department of Anatomy, Andhra Medical College, Vizagapatam*

In the course of the study of the nervous system of south Indian foetuses the level of termination of the spinal cord has been noted. Table 1 shows the vertebral level at which it was found to end in a series of foetuses of different ages.

Table 1

Sex	C.R. length (cm.)	Lower limit of spinal cord	Sex	C.R. length (cm.)	Lower limit of spinal cord
M	5·1	S 4	M	28·5	L 1
F	6·2	S 4	M	29·0	L 2
M	7·0	S 3	F	29·0	L 2
F	12·7	L 4	F	...	L 1
F	14·3	L 3	F	32·0	L 2
M	21·4	L 1	M	32·0	L 2
M	25·0	L 2	M	33·0	L 1
F	28·0	L 1	F	35·0	L 2
M	28·0	L 2	M	36·0	L 2
F	28·0	L 2	M	1 month (postnatal)	L 1

REVIEWS

Human Embryology and Morphology. By Sir Arthur Keith. 6th ed. (Pp. xii + 690; 578 figures; demy 8vo; 40*s*. net.) London: Edward Arnold and Co.

Sir Arthur Keith in the Preface to this 6th edition of his well-known book on Morphology and Embryology compares it with the 1st edition of 1901. He notes that in the intervening years the centre of embryological and morphological research has shifted from Germany to the United States, and he generously acknowledges the debt that the book owes to the Carnegie Laboratory in Baltimore. It is a pleasure to notice too his recognition of the immense amount of original work that constitutes Prof. Frazer's *Manual of Embryology*, to which also the present book owes much.

Sir Arthur gives an interesting review of his own changed attitude with regard to the teaching of Embryology. At one time 'clinical utility was the criterion employed', but now 'everything which seemed to throw new light on our understanding of the human body was welcomed to my pages'. But he has remained firm in his guiding principle 'that Embryology becomes a profitable study only when we interpret its events in the light of evolution'.

This principle has clearly been adhered to, for almost no matter where one opens the book a reference is found to lower vertebrates.

The book is hardly recognizable as the child born in 1901. Like the rest of us it has suffered from the war, and the binding, paper and print though surprisingly good are not equal to those of the 1st edition. The greatest change is in the 'Notes and References' appended to each chapter, and these are even increased from the 5th edition of 1933, comprising now nearly 50 pages of close-set type. Still, there are familiar features, the same friendly conversational style of writing, the same technique of line and stipple in the figures, many of the original blocks still being used.

Many of the figures have been borrowed, and not all lend themselves to the style of technique referred to. Several of the figures are printed so darkly that it is hard to make out the points they are meant to illustrate.

The book brings to mind the question as to how far students are prepared to read outside their set books. If they are so inclined, here is the book to show them where to browse, but, the curriculum being what it is and the tendency being to cut down to a bare minimum the time allotted to Anatomy, it is doubtful if many of them will have time to reap the rich harvest that Sir Arthur has prepared for them. There will be many sources of information that the students' teachers may have overlooked, but I suggest there are few important papers that have escaped Sir Arthur's comb. He is to be congratulated on the care with which he has ensured that the book shall be up to date. The task of collecting such a multitude of references is one which many a younger man would have shirked, but Sir Arthur has not only achieved this but has found the time and energy to give us his own opinions on many points, and has not hesitated to show us how these have changed with the passage of time and in face of new facts.

The child of 1901 has now become a book of reference that may be profitably studied both by students and their teachers.

C. M. WEST

Living Anatomy. A Photographic Atlas of Muscles in Action and Surface Contours. By R. D. Lockhart, M.D., Ch.M. (Pp. 71; 149 figures; royal 8vo; 12*s*. 6*d*. net.) London: Faber and Faber.

The aim of this book 'to awaken the student's interest in studying, literally at first hand, muscles in action in the living body' is excellent. The static representation by still photography of a dynamic subject is not easy, but by selecting examples where the

muscles are in isometric contraction, the author has presented the subject well. A brief description is given with most of the 149 photographs and the student should have little difficulty in recognizing similar features in his fellows and himself.

A few of the positions adopted by the subjects are unusual and it would have been better to omit them. The inclusion of Harvey's experiments to demonstrate the action of valves in veins is an excellent contribution, but the photographs should be placed in the book so as to give the impression of the action of gravity.

The photography is, on the whole, so good that isolated pictures which are not perfect stand out in contrast. This is particularly noticeable in Fig. 25 where, in spite of the author's warning in the Introduction, the lower border of the trapezius is shown running in the direction of the acromion process.

The book is worthy of its aim and should be very useful to medical students and others who must be able to recognize muscle activity in the living.

J. WHILLIS

IN MEMORIAM

CHARLES JOSEPH PATTEN, M.A., M.D., Sc.D., F.R.A.I.

Emeritus Professor of Anatomy, University of Sheffield

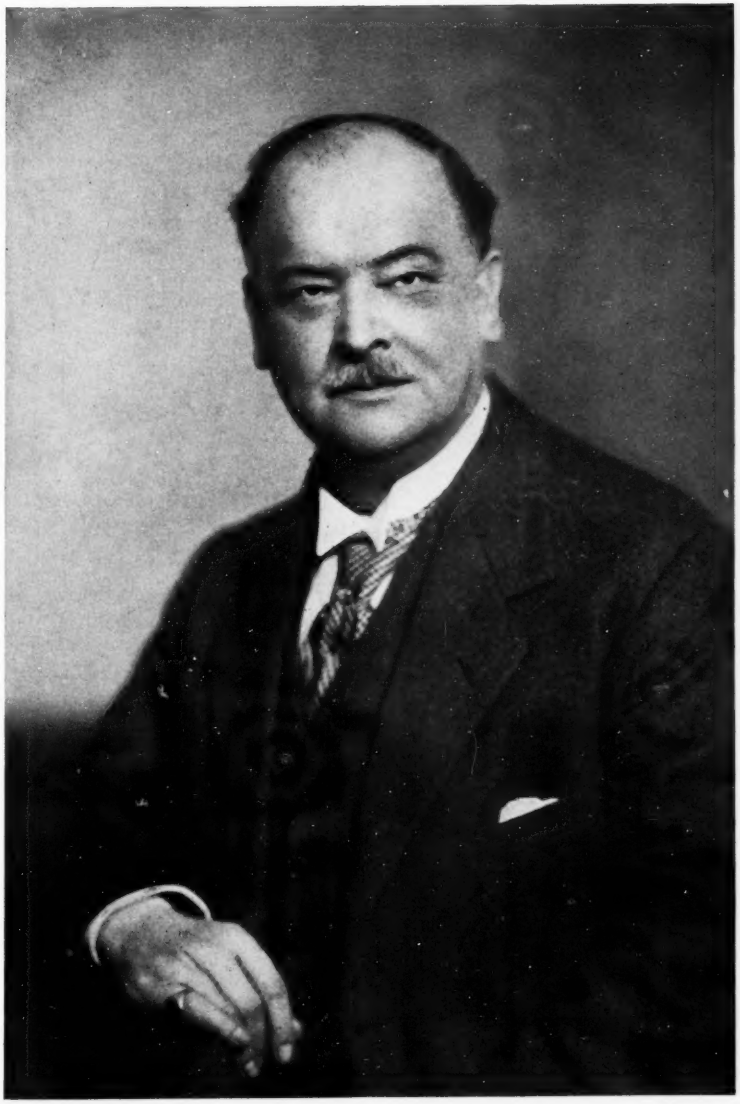
Charles Joseph Patten died on 13 June 1948, after a very brief illness, at his home in Farnham, Surrey, where he had moved from Sheffield only a year previously. In his later years he suffered from a severe affection of his eyes. By his death the Anatomical Society has lost one of its oldest members. He was the son of Richard Patten, of the Court of Exchequer, Ireland, and was born at Ballybrack, Co. Dublin, in 1870. Patten was educated at the High School, Dublin and later at Trinity College, Dublin, where he took a triple first in the Senior Moderatorship with a gold medal in Natural Science. He graduated in 1896 and became a Demonstrator in Anatomy in Cunningham's department at Trinity College. His research during his tenure of this office included studies of the topographical relations and surface anatomy of the heart, lungs and pleural cavities.

In 1901 he was elected Professor of Anatomy in the University of Sheffield (then a university college) in succession to Christopher Addison, now Lord Addison of Stallingborough. On retirement from his chair in 1935 he was given the title of Professor Emeritus. At Sheffield he developed the beautiful museum in the Anatomy department; his anatomical studies, on which he contributed many papers to the meetings of the Anatomical Society, were mainly in the field of physical anthropology, particularly variations in the osteology of the skull in primates and anomalies in human myology. He took a keen interest in finding the best methods for the preservation of the cadaver; his 'dry-tank' preservation has stood the exacting test of time. But his greatest interest over many years was the study of bird life and it was as an ornithologist that he became best known to the general public through his writings, lectures and broadcasts. Before leaving Sheffield he gave his excellent collection of bird skins to the Weston Park Museum, Sheffield. He had a great affection for birds and studied them mainly as a naturalist; it was their ways and habits, their nesting and particularly the problem of their migration that appealed most to him. He spent many weeks during university vacations in the off-shore lighthouses around the Irish coast studying bird migration and he wrote numerous papers and two books on bird life, one of which, *The Aquatic Birds of Great Britain*, published in 1912, is a standard work.

Patten acted as external examiner in anatomy at various times in the Universities of Wales, Birmingham, Leeds and Manchester and the Queen's University of Belfast. Most notable of Patten's qualities were his imperturbable cheerfulness, his generosity, tolerance, ready help and pronounced sense of humour. He had the gift of excellent mimicry and his lectures were enriched by his imitation of the sound and song of numerous birds. He was a devoted disciple of Charles Darwin whom he literally worshipped. He will be missed by a wide circle of friends. He married Mabel Elizabeth, daughter of Canon W. J. King, and is survived by his widow and a son and daughter, to whom we extend our deepest sympathy.

F. D.

M. A. MacC.



CHARLES JOSEPH PATTEN

ARTHUR ROBINSON, M.D., F.R.C.S. Edin. and Eng., LL.D.
1862–1948

Emeritus Prof. Arthur Robinson, who occupied the Chair of Anatomy in the University of Edinburgh from 1909 to 1931, died at Eastbourne on 3 December 1948, aged 86.

Arthur Robinson was born at Manchester in 1862, and he graduated M.B., C.M., with honours, at Edinburgh in 1883. After a brief apprenticeship in the teaching of Anatomy at Surgeons' Hall, he was appointed Demonstrator in the University Department by Prof. Turner. Next year he accepted a call to Owens College, Manchester, as Assistant to Prof. Morison Watson; and in 1888 he became Senior Demonstrator and, later, Lecturer in the Victoria University under Prof. A. H. Young.

The 70's and 80's were indeed vintage years for Edinburgh anatomists: Robinson himself (graduating two years before Harold J. Stiles—a surgeon-anatomist of the first rank) came in the midst and was the last survivor of an unparalleled group that included Morison Watson, J. H. Scott, D. J. Cunningham, A. H. Young, Johnson Symington, Arthur Thomson, David Hepburn, A. M. Paterson, J. T. Wilson, Robert Howden, James Musgrove, T. H. Bryce and Edward Fawcett—all of them Turner's Assistants and all of them professors in due season.

In 1896 the Medical School of The Middlesex Hospital decided to appoint a whole-time Lecturer to succeed Bland Sutton; and Robinson, with the reputation he had made in Manchester as teacher and investigator, was selected for that new post. His success in reorganizing and extending the Department at The Middlesex Hospital led to his appointment four years later to the Chair of Anatomy in King's College; and at similar short intervals he moved on—first, in 1905, to the University of Birmingham (where he was also Sub-Dean of the Faculty of Medicine), and finally to Edinburgh in 1909 to occupy the Chair left vacant by the early death of D. J. Cunningham. In all these posts, while continuing to investigate problems in his chosen fields of research, he abundantly displayed his administrative talent and his powers as a teacher.

In London, Robinson not only added to his reputation as an effective teacher—his classes in preparation for the 'Primary Fellowship Examination' spread his fame far and wide—but he also took an active part in the development of anatomical teaching in relation to the University of London. He served as Secretary of the Board of Intermediate Medical Studies, and he was particularly concerned with the movement that resulted in the formation of the Board of Studies in Human Anatomy and Morphology by the University Senate.

Robinson's association with Turner, who had himself made numerous contributions to the subject of placentation, and with A. H. Young, with whom he collaborated in several morphological studies, doubtless influenced his choice of Comparative Embryology and Vascular Morphology for his principal researches. During his Manchester period he was actively engaged in both of these fields, as the list of his published papers testifies, in addition to other developmental and morphological studies. His first paper appeared in 1887; and in 1890 he was awarded the

Gold Medal of the University of Edinburgh for his M.D. thesis 'Observations on the development of two rodents'. His interest in the development and morphology of veins, on which he came to be recognized as an authority, dates from 1891, when he contributed a paper to the first volume of Studies in Anatomy from Owens College. But the outstanding papers of the Manchester period are the two that appeared in the *Quart. J. micr. Sci.* on early developmental stages. These papers gave him his initial reputation as an embryologist, and it may be noted that he was asked in 1894 to give a Course of Lectures on Comparative Embryology as deputy for Milnes Marshall.

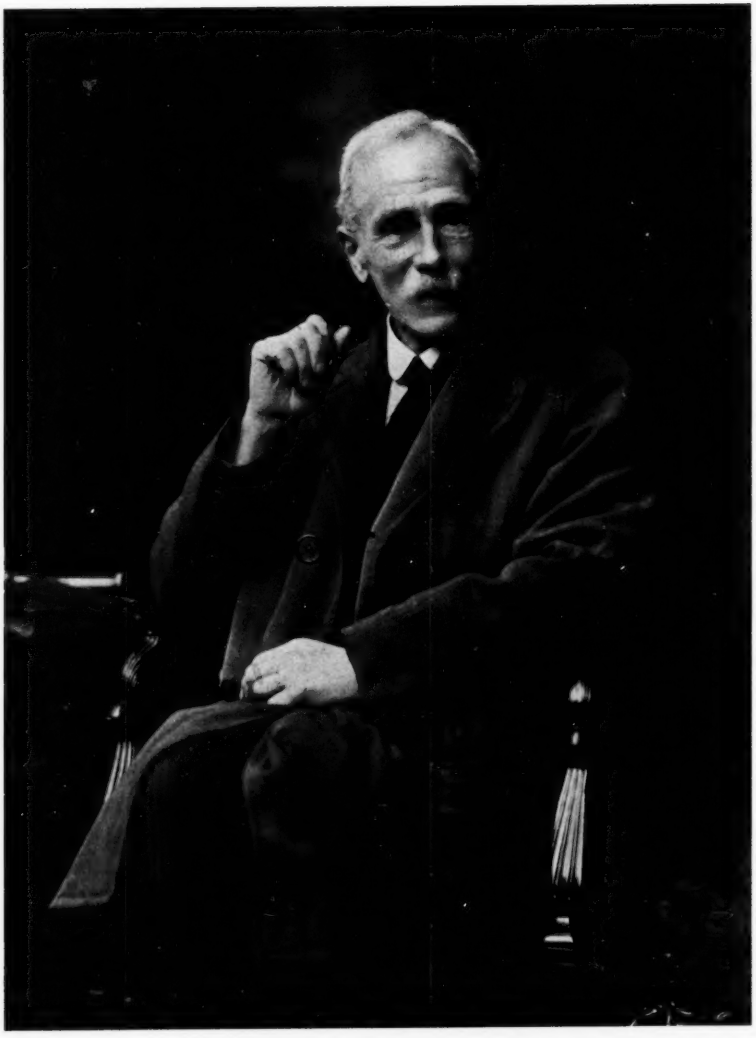
That reputation was firmly established by his better-known work on the Comparative Anatomy of the Placenta (*J. Anat., Lond.*, 1904, **38**), based on three lectures which he delivered in 1903 as Hunterian Professor of the Royal College of Surgeons of England.

In 1898 Robinson contributed several chapters to the second edition of the text-book edited by Henry Morris of the Middlesex Hospital; and in 1902 he resumed his collaboration with A. H. Young by contributing with him to the first edition of the *Text-Book of Anatomy* edited by D. J. Cunningham, in which they appeared as joint-authors of the Sections on 'General Embryology' and the 'Vascular System'. When Robinson succeeded Cunningham in the Edinburgh Chair he also succeeded him as editor of the *Text-Book* and became responsible, too, for successive editions of Cunningham's *Manual of Practical Anatomy*. He at once introduced the B.N.A. system of nomenclature on the grounds 'firstly, and chiefly, because it is more regular and definite than that hitherto in use, and, therefore, it tends to inculcate greater definiteness of idea and statement. Secondly, because it has been very generally adopted in Canada, Australia and America....' He thus brought to an issue the apparently interminable terminological arguments which were ultimately settled, so far as British anatomists are concerned, by the adoption of the Birmingham Revision in 1933.

These editorial labours, together with the heavy responsibilities of a large Department, took up a great deal of his time. Nevertheless, with his abounding energy, he continued to pursue the developmental problems that interested him; and his paper on 'The formation, rupture and closure of ovarian follicles' (1918) was a major contribution to the subject, in which he described the formation of 'secondary liquor folliculi' as the immediate cause of the rupture of the follicle.

In 1912 Robinson had been elected a Fellow of the Royal College of Surgeons of Edinburgh, and in 1920 he was Struthers Lecturer of that College. He took as his subject 'Prenatal Death', and he reviewed the evidence for the varying viability of the zygote in different animals with reference to the high rate of early abortion. On his transfer to Edinburgh he had been appointed Chairman of the William Ramsay Henderson Trust, and he was largely responsible for the series of Lectures inaugurated in 1924 by Prof. Elliot Smith who delivered a discourse on 'The Old and the New Phrenology'.

Robinson was a Fellow of King's College, London; and in 1924 he was elected to the Fellowship of the Royal College of Surgeons of England as a Member of twenty years' standing. The Royal Society of Edinburgh awarded him the Neill Prize for the period 1925–7 for his 'Contributions to Comparative Anatomy and



(Portrait by A. Swan Watson, Edinburgh)

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Embryology', and on his retirement he received the honorary degree of LL.D. from the University of Edinburgh. He joined the Anatomical Society in 1889, and he was President from 1920 to 1922.

Robinson was a man of great vigour, energy and enthusiasm. His brisk and agile movements earned him the name of 'Dancing Arthur' from his students, and they were an outward sign of the activity of his mind. With him, it was 'early to bed and early to rise'; and he did not spare himself in his professorial duties, in his duties as examiner (in which he had extensive experience), and in his long hours over his microscope.

His preparations for retirement were clouded by serious eye-trouble which gave rise to much anxiety and modified his intended activities. In the summer of 1931, through detachment of the retina, he lost the sight of one eye; and he had to wait nearly a year before a developing cataract in the other was ripe for treatment. Happily, the sight of that eye was then restored; and during his retirement, which he spent in the south of England, he was able to satisfy his catholic taste in literature by a greater measure of the general reading which even during his active professional life he had not neglected. He had to give up the golf which he had played with zest during his Birmingham and Edinburgh periods—except during the 1914–18 War when he characteristically refused to touch a club—but he was able for a time to indulge in long walks with still vigorous stride; and he enjoyed the less strenuous pursuit, dear to many retired professional men, of gardening.

Prof. Robinson married Emily, third daughter of John Baily, in 1888; and we extend our sympathy to Mrs Robinson in the loss she has sustained in the year of their Diamond Anniversary.

J. C. B.

E. B. J.

BIBLIOGRAPHY

1887 On the position and peritoneal relations of the mammalian ovary. *J. Anat. Physiol.* **21**, 169.

1888–9 On the anatomy of *Hyaena striata*. (With A. H. Young.) Parts I and II. *J. Anat. Physiol.* **23**, 90, 187.

1889 Observations on the earlier stages in the development of the lungs of rats and mice. *J. Anat. Physiol.* **23**, 224.

1890 Observations on the development of two rodents. M.D. Thesis, University of Edinburgh.

1891 The development of the posterior columns, of the posterior fissure and of the central canal of the spinal cord. *Studies in Anatomy from the Anatomical Department of the Owens College*, **1**, 67. Manchester: Cornish.

1891 Abnormalities of the venous system and their relation to the development of veins. *Studies in Anatomy from the Anatomical Department of the Owens College*, **1**, 197. Manchester: Cornish.

1891 Some points in the early development of *Mus musculus* and *Mus decumanus*. The relation of the yolk sac to the decidua and the placenta. *Rep. Brit. Ass.* p. 690. London: John Murray (1892).

1891 The formation and fate of the primitive streak, with observations on the archenteron and germinal layers of *Rana temporaria*. (With R. Assheton.) *Quart. J. micr. Sci.* **32**, 451.

1892 Observations upon the development of the segmentation cavity, the archenteron, the germinal layers, and the amnion in mammals. *Quart. J. micr. Sci.* **33**, 369.

1892 The nutritive importance of the yolk sac. *J. Anat. Physiol.* **26**, 308.

1893 Observations upon the development of the common ferret, *Mustela ferox*. *Anat. Anz.* **8**, 116.

1896 On the formation and structure of the optic nerve, and its relation to the optic stalk. *J. Anat. Physiol.* **30**, 319.

1898 The development and morphology of the vascular system in mammals. I. The posterior end of the aorta and the iliac arteries. (With A. H. Young.) *Proc. Roy. Soc.* **62**, 350; and *Studies in Anatomy from the Anatomical Department of the Owens College*, **2**, 1. Manchester: Cornish (1900).